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(54) Title: EXPRESSION OF GROUP B NEISSERIA MENINGITIDIS OUTER MEMBRANE (MB3) PROTEIN FROM YEAST AND VACCINES

(57) Abstract

The present invention relates, in general, to a method for obtaining the outer membrane protein meningococcal group B porin proteins, in particular MB3, and fusion proteins thereof. In particular, the present invention relates to a method of expressing the outer membrane protein meningococcal group B porin proteins in yeast. The invention also relates to a method of high level expression of the above-mentioned proteins wherein the rate of protein expression is enhanced by substituting a nucleotide sequence for the 5' region of the gene encoding said protein wherein the sequence has been optimized for yeast codon usage. The invention also relates to a vaccine comprising group A meningococcal polysaccharide (GAMP), group B meningococcal polysaccharide (GBMP) and group C meningococcal polysaccharide (GCMP) antigens, together with a pharmaceutically acceptable carrier. The invention also relates to a method of inducing an immune response in a mammal, comprising administering the above-mentioned vaccine to a mammal in an amount sufficient to induce an immune response.

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Expression of Group B *Neisseria meningitidis* Outer Membrane (MB3) Protein from Yeast and Vaccines

Background of the Invention

Field of the Invention

The present invention is in the field of recombinant genetics, protein expression, and vaccines. The present invention relates to a method of expressing in a recombinant yeast host an outer membrane group B porin protein from *Neisseria meningitidis*. The invention also relates to a vaccine comprising group A meningococcal polysaccharide (GAMP), group B meningococcal polysaccharide (GBMP) and group C meningococcal polysaccharide (GCMP) antigens, together with a pharmaceutically acceptable carrier. The invention also relates to a method of inducing an immune response in a mammal, comprising administering the above-mentioned vaccine to a mammal in an amount sufficient to induce an immune response.

Background Information

Meningococcal meningitis remains a worldwide problem, and occurs in both endemic and epidemic forms (Peltola, H., *Rev. Infect. Dis.* 5:71-91 (1983); Gotschlich, E.C., "Meningococcal Meningitis," in *Bacterial Vaccines*, Germanier, E., ed., Academic, New York (1984), pp.237-255). Epidemic disease occurs in

all parts of the world and incidences as high as 500 per 100,000 population have been reported. Without antibiotic treatment the mortality is extremely high (85%), and even with antibiotics, it remains at approximately 10%. In addition, patients cured by antibiotic therapy can still suffer serious and permanent neurologic deficiencies. These facts together with the emergence of sulfadiazine-resistant strains of *Neisseria meningitidis* promoted the rapid development of a commercial vaccine (Peltola, H., *Rev. Infect. Dis.* 5:71-91 (1983)).

Neisseria meningitidis is a gram-negative organism that has been classified serologically into groups A, B, 29e, W135, X, Y, and Z (Gotschlich, E.C., "Meningococcal Meningitis," in *Bacterial Vaccines*, Germanier, E., ed., Academic, New York (1984), pp.237-255). Additional groups H, I, and K were isolated in China (Ding, S.-Q. *et al., J. Biol. Stand.* 9:307-315 (1981)) and group L was isolated in Canada (Ashton, F.E. *et al., J. Clin. Microbiol.* 17:722-727 (1983)). The grouping system is based on the organisms' capsular polysaccharides. It was established (Lui, T.-Y. *et al., J. Biol. Chem.* 246:2849-2858 (1971)) that the group A polysaccharide is a partially O-acetylated (1→6) linked homopolymer of 2-acetamido-2-detoxy-D-mannopyranosyl phosphate, and that both groups B and C polysaccharides are homopolymers of sialic acid.

N. meningitidis groups A, B, and C are responsible for approximately 90% of cases of meningococcal meningitis. Success in the prevention of group A and C meningococcal meningitis was achieved using a bivalent polysaccharide vaccine (Gotschlich, E.C. *et al., J. Exp. Med.* 129:1367-1384 (1969); Artenstein, M.S. *et al., N. Engl. J. Med.* 282:417-420 (1970)); this vaccine became a commercial product and has been used successfully in the last decade in the prevention and arrest of major meningitis epidemics in many parts of the world. However, there has been a need to augment this vaccine because a significant proportion of cases of meningococcal meningitis are due to groups other than A and C. Group B is of particular epidemiologic importance, but groups Y and W135 are also significant (Cadoz, M. *et al., Vaccine* 3:340-342 (1985)). The inclusion of the group B polysaccharide in the vaccine has been a special problem

(see below); however, a tetravalent vaccine comprising groups A, C, W135, and Y has proven to be safe and immunogenic in humans (Cadoz, M. *et al.*, *Vaccine 3*:340-342 (1985)) and is the currently used meningococcal meningitis vaccine (Jennings, H.J., "Capsular Polysaccharides as Vaccine Candidates," in *Current Topics in Microbiol. and Immunol.*, Jann, D. and Jann, B., eds, Springer-Verlag, Berlin (1990) Vol 150:97-127).

The outer membranes of *Neisseria* species much like other Gram negative bacteria are semi-permeable membranes which allow free flow access and escape of small molecular weight substances to and from the periplasmic space of these bacteria but retard molecules of larger size (Heasley, F.A., *et al.*, "Reconstitution and characterization of the *N. gonorrhoeae* outer membrane permeability barrier," in *Genetics and Immunobiology of Neisseria gonorrhoeae*, Danielsson and Normark, eds., University of Umea, Umea, pp. 12-15 (1980); Douglas, J.T., *et al.*, *FEMS Microbiol. Lett. 12*:305-309 (1981)). One of the mechanisms whereby this is accomplished is the inclusion within these membranes of proteins which have been collectively named porins. These proteins are made up of three identical polypeptide chains (Jones, R.B., *et al.*, *Infect. Immun. 30*:773-780 (1980); McDade, Jr. and Johnston, *J. Bacteriol. 141*:1183-1191 (1980)) and in their native trimer conformation, form water filled, voltage-dependent channels within the outer membrane of the bacteria or other membranes to which they have been introduced (Lynch, E.C., *et al.*, *Biophys. J. 41*:62 (1983); Lynch, E.C., *et al.*, *Biophys. J. 45*:104-107 (1984); Young, J.D.E., *et al.*, *Proc. Natl. Acad. Sci. USA 80*:3831-3835 (1983); Mauro, A., *et al.*, *Proc. Natl. Acad. Sci. USA 85*:1071-1075 (1988); Young, J.D., *et al.*, *Proc. Natl. Acad. Sci. USA 83*:150-154 (1986)). Because of the relative abundance of these proteins within the outer membrane, these protein antigens have also been used to subgroup both *Neisseria gonorrhoeae* and *Neisseria meningitidis* into several serotypes for epidemiological purposes (Frasch, C.E., *et al.*, *Rev. Infect. Dis. 7*:504-510 (1985); Knapp, J.S., *et al.*, "Overview of epidemiological and clinical applications of auxotype/serovar classification of *Neisseria gonorrhoeae*," *The Pathogenic*

Neisseriae, Schoolnik, G.K., ed., American Society for Microbiology, Washington, pp. 6-12 (1985)). To date, many of these proteins from both gonococci and meningococci have been purified (Heckels, J.E., *J. Gen. Microbiol. 99*:333-341 (1977); James and Heckels, *J. Immunol. Meth. 42*:223-228 (1981); Judd, R.C., *Anal. Biochem. 173*:307-316 (1988); Blake and Gotschlich, *Infect. Immun. 36*:277-283 (1982); Wetzler, L.M., *et al.*, *J. Exp. Med. 168*:1883-1897 (1988)), and cloned and sequenced (Gotschlich, E.C., *et al.*, *Proc. Natl. Acad. Sci. USA 84*:8135-8139 (1987); McGuinness, B., *et al.*, *J. Exp. Med. 171*:1871-1882 (1990); Carbonetti and Sparling, *Proc. Natl. Acad. Sci. USA 84*:9084-9088 (1987); Feavers, I.M., *et al.*, *Infect. Immun. 60*:3620-3629 (1992); Murakami, K., *et al.*, *Infect. Immun. 57*:2318-2323 (1989); Wolff and Stern, *FEMS Microbiol. Lett. 83*:179-186 (1991); Ward, M.J., *et al.*, *FEMS Microbiol. Lett. 73*:283-289 (1992)).

The porin proteins were initially co-isolated with lipopolysaccharides (LPS). Consequently, the porin proteins have been termed "endotoxin-associated proteins" (Bjornson *et al.*, *Infect. Immun. 56*:1602-1607 (1988)). Studies on the wild type porins have reported that full assembly and oligomerization are not achieved unless LPS from the corresponding bacterial strain is present in the protein environment (Holzenburg *et al.*, *Biochemistry 28*:4187-4193 (1989); Sen and Nikaido, *J. Biol. Chem. 266*:11295-11300 (1991)).

The meningococcal porins have been subdivided into three major classifications which in antedated nomenclature were known as Class 1, 2, and 3 (Frasch, C.E., *et al.*, *Rev. Infect. Dis. 7*:504-510 (1985)). Each meningococcus examined has contained one of the alleles for either a Class 2 porin gene or a Class 3 porin gene but not both (Feavers, I.M., *et al.*, *Infect. Immun. 60*:3620-3629 (1992)); Murakami, K., *et al.*, *Infect. Immun. 57*:2318-2323 (1989)). The presence or absence of the Class 1 gene appears to be optional. Likewise, all probed gonococci contain only one porin gene with similarities to either the Class 2 or Class 3 allele (Gotschlich, E.C., *et al.*, *Proc. Natl. Acad. Sci. USA 84*:8135-8139 (1987); Carbonetti and Sparling, *Proc. Natl. Acad. Sci. USA 84*:9084-9088

(1987)). *N. gonorrhoeae* appear to completely lack the Class 1 allele. The data from the genes that have been thus far sequenced would suggest that all neisserial porin proteins have at least 70% homology with each other with some variations on a basic theme (Feavers, I.M., *et al.*, *Infect. Immun. 60*:3620-3629 (1992)). It has been suggested that much of the variation seen between these neisserial porin proteins is due to the immunological pressures brought about by the invasion of these pathogenic organisms into their natural host, man. However, very little is known about how the changes in the porin protein sequence effect the functional activity of these proteins.

It has been previously reported that isolated gonococcal porins of the Class 2 allelic type behave electrophysically somewhat differently than isolated gonococcal porins of the Class 3 type in lipid bilayer studies both in regards to their ion selectivity and voltage-dependence (Lynch, E.C., *et al.*, *Biophys. J. 41*:62 (1983); Lynch, E.C., *et al.*, *Biophys. J. 45*:104-107 (1984)). Furthermore, the ability of the different porins to enter these lipid bilayers from intact living bacteria seems to correlate not only with the porin type but also with the neisserial species from which they were donated (Lynch, E.C., *et al.*, *Biophys. J. 45*:104-107 (1984)). It would seem that at least some of these functional attributes could be related to different areas within the protein sequence of the porin. One such functional area, previously identified within all gonococcal Class 2-like proteins, is the site of chymotrypsin cleavage. Upon chymotrypsin digestion, this class of porins lack the ability to respond to a voltage potential and close. Gonococcal Class 3-like porins as well as meningococcal porins lack this sequence and are thus not subject to chymotrypsin cleavage but nonetheless respond by closing to an applied voltage potential (Greco, F., "The formation of channels in lipid bilayers by gonococcal major outer membrane protein," thesis, The Rockefeller University, New York (1981); Greco, F., *et al.*, *Fed. Proc. 39*:1813 (1980)).

As the *Neisseria* porins are among the most abundant proteins present in the outer membrane of these organisms, and as they do not undergo antigenic

shift during infection (unlike several other major surface antigens), their universal presence in both *Neisseria meningitidis* and *Neisseria gonorrhoea*, as well as their exposure at the surface, make them candidates for components of vaccines against these organisms. Patients convalescing from meningococcal disease produce anti-porin antibodies, and antibodies elicited by immunization with porin proteins are bactericidal to homologous serotypes. Furthermore, within a particular epidemiologic setting, most strains causing meningococcal disease belong to a limited number of serotypes, notably serotype 2 among strains with a class 2 protein and serotype 15 among strains with class 3 proteins. Therefore, class 2 and 3 proteins are attractive candidates for vaccines.

The major impediment for such studies has been the ability to easily manipulate the porin genes by modern molecular techniques and obtain sufficient purified protein to carry out the biophysical characterizations of these altered porin proteins. It was early recognized that cloned neisserial porin genes, when expressed in *Escherichia coli*, were lethal to the host *E. coli* (Carbonetti and Sparling, *Proc. Natl. Acad. Sci. USA 84*:9084-9088 (1987); Carbonetti, N.H., *et al.*, *Proc. Natl. Acad. Sci. USA 85*:6841-6845 (1988); Barlow, A.K., *et al.*, *Infect. Immun. 55*:2734-2740 (1987)). Thus, many of these genes were cloned and sequenced as pieces of the whole gene or placed into low copy number plasmids under tight expression control (Carbonetti, N.H., *et al.*, *Proc. Natl. Acad. Sci. USA 85*:6841-6845 (1988)). Under these conditions, even when the entire porin gene was expressed, very little protein accumulated that could be further purified and processed for characterization.

Another tack to this problem which has met with a modicum of success has been to clone the porin genes into a low copy, tightly controlled expression plasmid, introduce modifications to the porin gene, and then reintroduce the modified sequence back into *Neisseria* (Carbonetti, N.H., *et al.*, *Proc. Natl. Acad. Sci. USA 85*:6841-6845 (1988)). However, this has also been fraught with problems due to the elaborate restriction endonuclease system present in

Neisseria, especially gonococci (Davies, J.K., *Clin. Microbiol. Rev. 2*:S78-S82 (1989)).

While a vaccine comprising neisserial porin has long been sought, an effective meningococcal polysaccharide vaccine which would give complete coverage to all serogroup organisms and to all humans is also needed. Several serious problems remain in the development of such a broad range polysaccharide vaccine. First, it has been established that although the group A and C polysaccharides are efficacious in adults and older children, their effectiveness in infants has only been marginal (Goldschneider, I., *et al., J. Infect. Dis. 128*:769-776 (1973); Gotschlich, E.C., *et al.*, "The Immune Responses to Bacterial Polysaccharides in Man," In: *Antibodies in Human Diagnosis and Therapy*, Haber, E. and Krause, R.M., eds., Raven, New York (1977), pp. 391-402). Second, the group B meningococcal polysaccharide is only poorly immunogenic in man (Wyle, F.A., *et al., J. Infect. Dis. 126*:514-521 (1972)). A third problem is the tendency for multivalent vaccines to be less immunogenic than each component would be if administered individually (Insel, R.A., "Potential alterations in immunogenicity by combining or simultaneously administering vaccine components," In: *Annals of the New York Academy of Sciences. Vol. 754. Combined Vaccines and Simultaneous Administration: Current Issues and Perspectives*, Williams, J.C., *et al.*, eds, New York Academy of Sciences, New York (1993), pp. 35-47; Clemens, J., *et al.*, "Interactions between PRP-T vaccine against *Haemophilus influenzae* type b and conventional infant vaccines: lessons for future studies of simultaneous immunization and combined vaccines," In: *Annals of the New York Academy of Sciences, Vol. 754. Combined Vaccines and Simultaneous Administration: Current Issues and Perspectives*, Williams, J.C., *et al.*, eds, New York Academy of Sciences, New York (1993), pp. 255-266; Paradiso, P.R., *et al., Pediatrics 92(6)*:827-832 (1993)).

Presently available vaccines against group A and C *N. meningitidis* are poorly immunogenic in human infants (age two and under) because, in contrast

to the immunity generated by most antigens, a polysaccharide-specific immune response in infants is T-cell-independent. In the absence of T-cell involvement, an immune response is of short duration. More importantly, no memory is demonstrable, so no "booster" reactions occur. Furthermore, antibody affinity maturation does not occur. These deficiencies are due to the inability of polysaccharides to specifically bind to T-cells. Presumably, the structural features required for association with a T-cell receptor do not exist in the majority of polysaccharides. Because of the T-cell independent nature of the immune response, the antibody response to a polysaccharide in infants is limited to antibodies of the IgM isotype; the isotype switching necessary for production of non-IgM antibodies requires T-cell involvement. Polysaccharide antigens present less of a problem in more mature humans (over age two), as they are able to induce antibodies of the IgG isotype as well as IgM (Yount *et al., J. Exp. Med. 127*:633-646 (1968)).

The group B meningococcal polysaccharide is even less immunogenic in humans of all ages than other polysaccharides. Two major explanations have been proposed to account for this characteristic (Jennings, H.J., *Adv. Carbohydr. Chem. Biochem. 41*:155-208 (1983); Lifely, M.R. *et al., Vaccine 5*:11-26 (1987)). One is that the group B meningococcal polysaccharide, an α-(2→8)-linked sialic acid homopolymer, is rapidly depolymerized in human tissue because of the action of neuraminidase. The other is that the structure is recognized as "self" by the human immune system and in consequence, the production of antibody specific for this structure is suppressed. The weight of evidence is in favor of the latter explanation because a neuraminidase-sensitive variant of the group C meningococcal polysaccharide [an α-(2→9)-linked sialic acid homopolymer] still proved to be highly immunogenic in man (Glode, M.P. *et al., J. Infect. Dis. 139*:52-59 (1979)). In addition it was demonstrated that conjugation of the group B polysaccharide to a protein carrier (tetanus toxoid) through its terminal nonreducing sialic acid, which stabilizes the polysaccharide to neuraminidase, did not result in any significant enhancement in its immunogenicity (Jennings, H.J.

and Lugowski, C., *J. Immunol. 127*:1011-1018 (1981)). The above observations are consistent with a theory that the immune mechanism avoids the production of antibody having a specificity for the α-(2→8)-linked sialic acid residues. This theory was further confirmed by the identification of α-(2→8)-linked sialic acid residues in the oligosaccharides of human and animal tissue. A novel approach to overcoming the poor immunogenicity of the group B polysaccharide has been to modify it chemically.

The T-cell independent quality of polysaccharide antigens in infant humans can be overcome by conjugating (covalently coupling) the polysaccharide to a protein carrier. The capsular polysaccharides of the bacteria primarily responsible for postneonatal meningitis have been conjugated to protein carriers: these include type b *H. influenzae* (Schneerson, R. *et al., J. Exp. Med. 152*:361-376 (1980); Anderson, P.W., *Infect. Immun. 39*:233-238 (1983); Marburg, S. *et al., J. Am. Chem. Soc. 108*:5282-5287 (1986)), group A (Jennings, H.J. and Lugowski, C., *J. Immunol. 127*:1011-1018 (1981)) ; Beuvery, E.C. *et al., Vaccine 1*:31-36 (1983)), B (Jennings, H.J. and Lugowski, C., *J. Immunol. 127*:1011-1018 (1981)), and C (Jennings, H.J. and Lugowski, C., *J. Immunol. 127*:1011-1018 (1981)); Beuvery, E.C. *et al., Infect. Immun. 40*:39-45 (1983)) *N. meningitidis*, and type 6A *Strep. pneumoniae* (Chu, C. *et al., Infect. Immun. 40*:245-256 (1983)). For the choice of carrier protein most investigators have used tetanus toxoid or diphtheria toxoid, two proteins currently used as infant vaccines. A recent innovation on this theme has been the use of a mutant-derived diphtheria toxin (CRM_{197}) (Anderson, P.W., *Infect. Immun. 39*:233-238 (1983)) which is nontoxic. The significance of this protein is that because it does not require detoxifying by treatment with formaldehyde, all its amino groups remain underivatized, which greatly facilitates the conjugation process.

The use of other potential bacterial proteins as carriers has not been extensively explored. However, a serotype outer member protein of *N. meningitidis* has been used as a protein carrier (Marburg, S. *et al., J. Am. Chem. Soc. 108*:5282-5287 (1986)).

In light of the foregoing, it will be clear that there is a significant need for a process by which large quantities of the outer membrane group B porin proteins of *N. meningitidis* can be obtained. It will also be clear that a need exists for a polysaccharide vaccine which would give complete coverage to the three major serogroups of *N. meningitidis*, groups A, B and C, and which would provide immunity against these organisms to both infants and more mature humans.

Summary of the Invention

It is a general object of the invention to provide a method of expressing in yeast the meningococcal group B porin proteins, in particular, the class 3 porin protein.

It is a specific object of the invention to provide a method of expressing the outer membrane meningococcal group B porin protein or a fusion protein thereof in yeast, comprising:

(a) cloning into a plasmid having a selectable marker a gene coding for a protein selected from the group consisting of:

(i) a mature porin protein

(ii) a fusion protein which is a mature porin protein fused to a yeast secretion signal peptide;

wherein said gene is operably linked to a yeast promoter;

(b) transforming said plasmid containing said gene into a yeast strain;

(c) selecting the transformed yeast by growing said yeast in a culture medium allowing selection of said transformed yeast;

(d) growing the transformed yeast, and

(e) inducing expression of said protein to give yeast containing said protein;

wherein the protein so expressed comprises more than about 2% of the total protein expressed in said yeast.

It is another specific object of the invention to provide a method of expressing a mature porin protein or fusion protein thereof, wherein the protein so expressed comprises about 3-5% of the total protein expressed in yeast.

It is yet another specific object of the invention to provide a method of expressing a mature porin protein wherein the protein is the *Neisseria meningitidis* outer membrane meningococcal group B porin protein (MB3).

It is another specific object of the invention to provide a method of expressing a mature porin protein or fusion protein thereof, wherein the yeast promoter is the AOX1 promoter.

It is another specific object of the invention to provide a method of expressing the outer membrane meningococcal group B porin protein or a fusion protein thereof in yeast, wherein the yeast secretion signal peptide is selected from the group consisting of the secretion signal of the *S. cerevisiae* α-mating factor prepro-peptide and the secretion signal of the *P. pastoris* acid phosphatase gene (*PHO*).

It is yet another specific object of the invention to provide a method of expressing MB3 or a fusion protein thereof in yeast as described above, wherein the plasmid is selected from the group consisting of pHIL-D2, pHIL-S1, pPIC9 and pPIC9K.

It is a further specific object of the invention to provide a method of expressing the above-described meningococcal group B porin protein or fusion protein wherein at least one codon of the 5′ region of the gene encoding said protein has been changed so as to be optimized for yeast codon usage.

It is still a further specific object of the invention to provide a method of expressing the above-described meningococcal group B porin protein or fusion protein wherein the 5′ region of the gene encoding said protein comprises a nucleotide sequence that incorporates codons optimized for *P. pastoris* codon usage.

It is another specific object of the invention to provide a method as described above wherein the codon changes are selected from the group of

changes consisting of: (GTT to GTC at positions 4-6 of the native sequence), (ACC to ACT at positions 7-9 of the native sequence), (CTG to TTG at positions 10-12 of the native sequence), (GGC to GGT at positions 16-18 of the native sequence), (ACC to ACT at positions 19-21 of the native sequence), (ATC to ATT at positions 22-24 of the native sequence), (AAA to AAG at positions 25-27 of the native sequence), (GCC to GCT at positions 28-30 of the native sequence), (GGC to GGT at positions 31-33 of the native sequence), (GTA to GTT at positions 34-36 of the native sequence), (GAA to GAG at positions 37-39 of the native sequence); wherein said positions are numbered from the first nucleotide of the native nucleotide sequence encoding said protein.

It is another specific object of the invention to provide a method as described above wherein the 5' region of the gene includes codons optimized for *P. pastoris* codon usage, and wherein the nucleotide sequence is SEQ ID NO: 26.

It is another specific object of the invention to provide a method of expressing the above-mentioned protein wherein the yeast secretes the protein or fusion protein.

It is another specific object of the invention to provide a method of expressing the above-mentioned protein wherein the vector from which the secreted protein is expressed is selected from the group consisting of pHIL-S1, pPIC9, and pPIC9K.

It is another specific object of the invention to provide a method of purifying insoluble, intracellular outer membrane meningococcal group B porin protein or fusion protein thereof obtained according to the invention comprising:

(a) lysing the yeast described above which has expressed the protein to release said protein as an insoluble membrane bound fraction;

(b) washing the insoluble material obtained in step (a) with buffers to remove contaminating yeast cellular proteins;

(c) suspending and dissolving said insoluble fraction obtained in step (b) in aqueous solution of denaturant;

(d) diluting the solution obtained in step (c) with a detergent: and

(e) purifying said protein by gel filtration and ion exchange chromatography.

It is another specific object of the invention to provide a method of purifying the outer membrane meningococcal group B porin protein or fusion protein thereof obtained according to the invention comprising:

(a) centrifuging the yeast culture described above which has expressed the protein to isolate the protein as soluble secreted material;

(b) removing contaminating yeast culture impurities from the soluble secreted material obtained in step (a) by precipitating said impurities with about 20% ethanol, wherein the soluble secreted material remains in the soluble fraction;

(c) precipitating the secreted material from the soluble fraction of step (b) with about 80% ethanol;

(d) washing the precipitated material obtained in step (c) with a buffer to remove contaminating yeast secreted proteins;

(e) suspending and dissolving the precipitated material obtained in step (d) in an aqueous solution of detergent; and

(f) purifying the protein by ion exchange chromatography.

It is a further specific object of the invention to provide a yeast host cell that contains a gene coding for a protein selected from the group consisting of:

(a) a mature porin protein

(b) a fusion protein which is a mature porin protein fused to a yeast secretion signal peptide;

wherein said gene is operably linked to a yeast promoter.

It is still another specific object of the invention to provide a yeast host cell as described above which is capable of expressing the *Neisseria meningitidis* mature outer membrane class 3 protein of serogroup B (MB3).

It is still another specific object of the invention to provide a yeast host cell as described above wherein the yeast promoter is the AOX1 promoter.

It is another object of the invention to provide a vaccine comprising group A meningococcal polysaccharide (GAMP), group B meningococcal polysaccharide (GBMP), and group C meningococcal polysaccharide (GCMP) antigens, together with a pharmaceutically acceptable carrier.

It is still another specific object of the invention to provide a method of inducing an immune response in a mammal, comprising administering to a mammal the above-described vaccine in an amount sufficient to induce an immune response in a mammal.

Further objects and advantages of the present invention will be clear from the description that follows.

Brief Description of the Drawings

Figure 1: A diagram showing the sequencing strategy of the *PorB* gene. The PCR product described in Example 1 (Materials and Methods section) was ligated into the *Bam*HI-*Xho*I site of the expression plasmid pET-17b. The initial double stranded primer extension sequencing was accomplished using oligonucleotide sequences directly upstream of the *Bam*HI site and just downstream of the *Xho*I site within the pET-17b plasmid. Additional sequence data was obtained by making numerous deletions in the 3′ end of the gene, using exonuclease III/mung bean nuclease reactions. After religation and transformation back into *E. coli*, several clones were selected on size of insert and subsequently sequenced. This sequencing was always from the 3′ end of the gene using an oligonucleotide primer just downstream of the *Bpu*11021 site.

Figure 2: A gel electrophoresis showing the products of the PCR reaction (electrophoresed in a 1% agarose using TAE buffer).

Figures 3A and 3B. Fig. 3A: SDS-PAGE analysis of whole cell lysates of *E. coli* hosting the control pET-17b plasmid without inserts and an *E. coli*

clone harboring pET-17b plasmid containing an insert from the obtained PCR product described in the materials and methods section. Both cultures were grown to an O.D. of 0.6 at 600 nm, IPTG added, and incubated at 37°C for 2 hrs. 1.5 mls of each of the cultures were removed, centrifuged, and the bacterial pellet solubilized in 100 µl of SDS-PAGE preparation buffer. Lane A shows the protein profile obtained with 10 µl from the control sample and Lanes B (5 µl) and C (10 µl) demonstrate the protein profile of the *E. coli* host expressing the PorB protein. Fig. 3B: Western blot analysis of whole cell lysates of *E. coli* harboring the control pET-17b plasmid without insert after 2 hrs induction with IPTG, Lane A, 20 µl and a corresponding *E. coli* clone containing a porB-pET-17b plasmid, Lane B, 5 µl; Lane C, 10 µl; and Lane D, 20 µl. The monoclonal antibody 4D11 was used as the primary antibody and the western blot developed as described. The pre-stained low molecular weight standards from BRL were used in each case.

Figure 4: The nucleotide sequence and the translated amino acid sequence of the mature *PorB* gene cloned into the expression plasmid pET-17b. The two nucleotides which differ from the previously published serotype 15 *PorB* are underlined.

Figure 5: A graph showing the Sephacryl S-300 column elution profile of both the wild type Class 3 protein isolated from the meningococcal strain 8765 and the recombinant Class 3 protein produced by BL21(DE3) -Δ*omp*A *E. coli* strain hosting the r3pET-17b plasmid as monitored by absorption at 280nm and SDS-PAGE analysis. The void volume of the column is indicated by the arrow. Fractions containing the meningococcal porin and recombinant porin as determined by SDS-PAGE are noted by the bar.

Figure 6: A graph showing the results of the inhibition ELISA assays showing the ability of the homologous wild type (wt) PorB to compete for reactive antibodies in six human immune sera. The arithmetic mean inhibition is shown by the bold line.

Figure 7: A graph showing the results of the inhibition ELISA assays showing the ability of the purified recombinant PorB protein to compete for reactive antibodies in six human immune sera. The arithmetic mean inhibition is shown by the bold line.

Figure 8: A graph showing a comparison of these two mean inhibitions obtained with the wt and recombinant PorB protein.

Figure 9A and 9B: The nucleotide sequence and the translated amino acid sequence of the mature class II porin gene cloned into the expression plasmid pET-17b.

Figure 10A and 10B: The nucleotide sequence and the translated amino acid sequence of the fusion class II porin gene cloned into the expression plasmid pET-17b.

Figure 11 (panels A and B): Panel A depicts the restriction map of the pET-17b plasmid. Panel B depicts the nucleotide sequence between the *BglII* and *XhoI* sites of pET-17b. The sequence provided by the plasmid is in normal print while the sequence inserted from the PCR product are identified in bold print. The amino acids which are derived from the plasmid are in normal print while the amino acids from the insert are in bold. The arrows demarcate where the sequence begins to match the sequence in Figure 4 and when it ends.

Figure 12: A graph showing the level of expression of MB3 for clone pnv 322, where the expression vector used is pHIL-D2. The level of MB3 expressed is depicted as mg of insoluble MB3 per gram of cell pellet per unit time.

Figure 13A: The DNA sequence and translated amino acid sequence of pNV15 (MB3 in pET24a) before codon preference optimization.

Figure 13B: The DNA sequence and translated amino acid sequence of Men.Class3 opt. (MB3 optimized for yeast codon preference).

Figures 14A and 14B: Graphs showing the elution of MB3 from a size exclusion column. MB3 expressed in an intracellular form was purified by a denaturation/renaturation protocol, followed by gel filtration and ion exchange chromatography. The resultant purified protein exhibited by size exclusion

chromatography an elution profile which resembles the recombinant class 3 protein overexpressed in *E. coli*, and both give the same elution profile as the native wild-type counterpart. This indicates that MB3 refolds and oligomerizes, achieving full native conformation. 14(A): the elution profile of MB3; 14(B): the elution profile of class 3 protein expressed and refolded from *E. coli* inclusion bodies.

Figure 15: A graph showing the size exclusion chromatography of purified MB3 on a Superose 12 (Pharmacia) column connected to an HPLC (Hewlett Packard model 1090). Based on the comparison of MB3 with the native wild-type counterpart, as well as calibration using molecular weight standards (designated as arrows), the elution profile is indicative of trimeric assembly. Molecular weight markers are: 1 = thyroglobulin (670,000); 2 = gammaglobulin (158,000); 3 = myoglobin (17,000).

Figures 16A, 16B and 16C: The DNA sequence of clone pnv 322. This clone has the MB3 gene inserted into the *EcoR*I site of the Invitrogen expression vector pHIL-D2. MB3 is thus inserted directly downstream from the *AOX1* promoter. This construct allows intracellular expression. Vector sequences are shown in upper case letters, while the MB3 sequence is given in lower case letters.

Figures 17A, 17B and 17C: The DNA sequence of clone pnv 318. This clone has the MB3 gene inserted into the *XhoI-BamHI* sites of the Invitrogen expression vector pHIL-S1. MB3 is thus inserted directly downstream from the *PHO1* leader peptide, in frame with the secretion signal open reading frame for secretion of expressed protein. Vector sequences are shown in upper case letters, while the MB3 sequence is given in lower case letters.

Figures 18A, 18B and 18C: The DNA sequence of clone pnv 342. This clone has the MB3 gene inserted into the *EcoR*I-*AvrII* sites of the Invitrogen expression vector pPIC-9. MB3 is thus inserted directly downstream from the secretion signal of α-factor prepro peptide, for secretion of expressed protein.

Vector sequences are shown in upper case letters, while the MB3 sequence is given in lower case letters.

Figures 19A, 19B and 19C: The DNA sequence of clone pnv 350. This clone has the MB3 gene inserted into the *EcoR*I-*Avr*II sites of the Invitrogen expression vector pPIC-9K. MB3 is thus inserted directly downstream from the secretion signal of α-factor prepro peptide, for secretion of expressed protein. Vector sequences are shown in upper case letters, while the MB3 sequence is given in lower case letters.

Figure 20: A graph showing the absorbance spectra (electropherogram) of GAMP, TT-monomer, and GAMP-TT conjugate.

Figure 21: A graph showing the absorbance spectra (electropherogram) of GCMP, TT-monomer, and GCMP-TT conjugate.

Figure 22: A graph showing the A-specific IgG ELISA titer elicited by monovalent (A) and trivalent (A/B/C) meningococcal conjugate vaccines in mice.

Figure 23: A graph showing the B-specific IgG ELISA titer elicited by monovalent (A) and trivalent (A/B/C) meningococcal conjugate vaccines in mice.

Figure 24: A graph showing the C-specific IgG ELISA titer elicited by monovalent (C) and trivalent (A/B/C) meningococcal conjugate vaccines in mice.

Figure 25: A graph showing the A-specific bacteriocidal activity elicited by monovalent (A) and trivalent (A/B/C) meningococcal conjugate vaccines in mice.

Figure 26: A graph showing the B-specific bacteriocidal activity elicited by monovalent (A) and trivalent (A/B/C) meningococcal conjugate vaccines in mice.

Figure 27: A graph showing the C-specific bacteriocidal activity elicited by monovalent (A) and trivalent (A/B/C) meningococcal conjugate vaccines in mice.

Detailed Description of the Invention

It is possible to overcome some of the difficulties involved in expressing and purifying the outer membrane group B porin proteins of *N. meningitidis* from *E. coli*. The DNA sequences of the mature porin proteins, e.g. class 2 and class 3 as well as fusions thereof, were amplified using the chromosome of the meningococcal bacteria as a template for the PCR reaction. The amplified porin sequences were ligated and cloned into an expression vector containing the T7 promoter. *E. coli* strain BL21 lysogenic for the DE3 lambda phage (Studier and Moffatt, *J. Mol. Biol. 189*:113-130 (1986)), modified to eliminate the *ompA* gene, was selected as one expression host for the pET-17b plasmid containing the porin gene. Upon induction, large amounts of the meningococcal porin proteins accumulated within *E. coli* without any obvious lethal effects to the host bacterium. The expressed meningococcal porin proteins were extracted and processed through standard procedures and finally purified by molecular sieve chromatography and ion exchange chromatography. As judged by the protein profile from the molecular sieve chromatography, the recombinant meningococcal porins eluted from the column as trimers. To be certain that no PCR artifacts had been introduced into the meningococcal porin genes to allow for such high expression, the inserted PorB gene sequence was determined. Inhibition ELISA assays were used to give further evidence that the expressed recombinant porin proteins had renatured into their natural antigenic and trimer conformation.

As an alternative to the bacterial *E. coli* host system, Meningococcal B Class 3 porin protein (MB3) may be expressed in yeast. A preferred host is the methylotrophic yeast *Pichia pastoris*, which may be transformed with the *Pichia* Expression Kit developed by Invitrogen. Yeasts are attractive hosts for the production of heterologous proteins. Unlike prokaryotic systems, their eukaryotic subcellular organization enables them to carry out many of the post-translational

folding, processing and modification events required to produce "authentic" and bioactive proteins. As a eukaryote, *Pichia pastoris* has many of the advantages of a higher eukaryotic expression system, while being as easy to manipulate as *E. coli* or *Saccharomyces cerevisiae*. As a yeast, it shares the advantages of molecular and genetic manipulations with *Saccharomyces*, and it has the added advantages of 10- to 100-fold higher heterologous protein expression levels and the protein processing characteristics of higher eukaryotes.

Expression in *Pichia* also provides advantages compared to expression in other yeast strains because *Pichia* does not tend to hyperglycosylate proteins as does *S. cerevisiae*. Further, proteins expressed and modified in *Pichia* may be more useful therapeutically than those produced by *S. cerevisiae*, as oligosaccharides added by *Pichia* lack the α1,3 glycan linkages which are believed to be primarily responsible for the hyper-antigenic nature of proteins produced by *S. cerevisiae*. Several vaccine antigens have been produced in yeast cells, including hepatitis B surface antigen which is in clinical use (Cregg *et al.*, *Bio/Technology 11*:905-910 (1993)).

Unlike the porin proteins of *E. coli* and a few other gram negative bacteria, relatively little is known about how changes in the primary sequence of porins from *Neisseria* effect their ion selectivity, voltage dependence, and other biophysical functions. Recently, the crystalline structure of two *E. coli* porins, OmpF and PhoE, were solved to 2.4Å and 3.0Å, respectively (Cowan, S.W., *et al.*, *Nature 358*:727-733 (1992)). Both of these *E. coli* porins have been intensively studied owing to their unusual stability and ease with which molecular genetic manipulations could be accomplished. The data obtained for the genetics of these two porins correlated well with the crystalline structure. Although it has been shown in several studies using monoclonal antibodies to select neisserial porins that the surface topology of *Neisseria* closely resembles that of these two *E. coli* porins (van der Ley, P., *et al.*, *Infect. Immun. 59*:2963-2971 (1991)), almost no information is available about how changes in amino acid sequences in specific areas of the neisserial porins effect their biophysical characteristics,

as is known about the *E. coli* porins (Cowan, S.W., *et al.*, *Nature 358*:727-733 (1992)).

Two of the major problems impeding this research are: (1) the inability to easily manipulate *Neisseria* genetically by modern molecular techniques and (2) the inability to express sufficient quantities of neisserial porins in *E. coli* or yeast for further purification to obtain biophysical and biochemical characterization data. In fact, most of the DNA sequence data on gonococcal and meningococcal porins have been obtained by cloning overlapping pieces of the porin gene and then reconstructing the information to reveal the entire gene sequence (Gotschlich, E.C., *et al.*, *Proc. Natl. Acad. Sci. USA 84*:8135-8139 (1987); Murakami, K., *et al.*, *Infect. Immun. 57*:2318-2323 (1989)). Carbonetti *et al.* were the first to clone an entire gonococcal porin gene into *E. coli* using a tightly controlled pT7-5 expression plasmid. The results of these studies showed that when the porin gene was induced, very little porin protein accumulated and the expression of this protein was lethal to the *E. coli* (Carbonetti and Sparling, *Proc. Natl. Acad. Sci. USA 84*:9084-9088 (1987)). In additional studies, Carbonetti *et al.* (*Proc. Natl. Acad. Sci. USA 85*:6841-6845 (1988)) did show that alterations in the gonococcal porin gene could be made in this system in *E. coli* and then reintroduced into gonococci. However, the ease with which one can make these manipulations and obtain enough porin protein for further biochemical and biophysical characterization seems limited.

Feavers *et al.* have described a method to amplify, by PCR, neisserial porin genes from a wide variety of sources using two synthesized oligonucleotides to common domains at the 5′ and 3′ ends of the porin genes respectively (Feavers, I.M., *et al.*, *Infect. Immun. 60*:3620-3629 (1992)). The oligonucleotides were constructed such that the amplified DNA could be forced cloned into plasmids using the restriction endonucleases *Bgl*II and *Xho*I.

Using the Feavers *et al.* PCR system, the DNA sequence of the mature PorB protein from meningococcal strain 8765 serotype 15 was amplified and ligated into the *Bam*HI-*Xho*I site of the T7 expression plasmid pET-17b. This

placed the mature PorB protein sequence in frame directly behind the T7 promoter and 20 amino acids of the ϕ10 protein including the leader sequence. Upon addition of IPTG to a culture of *E. coli* containing this plasmid, large amounts of PorB protein accumulated within the bacteria. A complete explanation for why this construction was non-lethal to the *E. coli* and expressed large amount of the porin protein, await further studies. However, one possible hypothesis is that by replacing the neisserial promoter and signal sequence with that of the T7 and ϕ10 respectively, the porin product was directed to the cytoplasm rather than toward the outer membrane. Henning and co-workers have reported that when *E. coli* OmpA protein and its fragments are expressed, those products which are found in the cytoplasm are less toxic than those directed toward the periplasmic space (Klose, M., *et al.*, *J. Biol. Chem. 263*:13291-13296 (1988); Klose, M., *et al.*, *J. Biol. Chem. 263*:13297-13302 (1988); Freudl, R., *et al.*, *J. Mol. Biol. 205*:771-775 (1989)). Whatever the explanation, once the PorB protein was expressed, it was easily isolated, purified and appeared to reform into trimers much like the native porin. The results of the inhibition ELISA data using human immune sera suggests that the PorB protein obtained in this fashion regains most if not all of the antigenic characteristics of the wild type PorB protein purified from meningococci. This expression system lends itself to the easy manipulation of the neisserial porin gene by modern molecular techniques. In addition, this system allows one to obtain large quantities of pure porin protein for characterization. In addition, the present expression system allows the genes from numerous strains of *Neisseria*, both gonococci and meningococci, to be examined and characterized in a similar manner.

The *Neisseria meningitidis* outer membrane class 3 protein from serogroup B (MB3) was also expressed in the methylotrophic yeast *Pichia pastoris* by placing the MB3 DNA fragment under the control of the strong *P. pastoris* alcohol oxidase promoter *AOX1*. Upon induction on methanol, strains of *P. pastoris* transformed with the recombinant plasmids produced either a native or a fusion MB3 protein, which were reactive with mouse polyclonal

antibodies raised against the wild type counterpart. In shaking flask cultures, engineered *P. pastoris* yielded about 1-3 mg of expressed protein per gram of pelleted wet cells, or 100-600 mg per liter, which corresponded to 10-15% of the yeast cell suspension or about 3-5% of total cellular proteins (Table 4). Full-length MB3 DNA was cloned into each of four Pichia Expression Vectors developed by Invitrogen. To obtain the expression of monomeric, full size 34 kDa MB3 protein, the intracellular pHIL-D2 vector was used. A map of the pHIL-D2 vector may be found on p. 19 of the Invitrogen Instruction Manual for the *Pichia* Expression Kit, Version E, the contents of which is hereby incorporated by reference. This construct provided maximal expression levels (up to 3 mg of MB3 per gram of cells) (Tables 3 and 4). The expressed product was not secreted, being mainly (95%) insoluble, and it was tightly associated with cell membranes.

To further increase the possibility for the secretion of expressed MB3, three other vectors with different secretion signals were also used: the vector pHIL-S1, which carries a native *Pichia pastoris* signal sequence from the acid phosphatase gene, *PHO1*, and the vectors pPIC9 and pPIC9K, which carry the secretion signal from the *S. cerevisiae* α-mating factor prepro-peptide. Maps of the pHIL-S1 and pPIC9 vectors may be found on pp. 21-22 of the Invitrogen Instruction Manual for the *Pichia* Expression Kit, Version E. It was found that the pHIL-S1/MB3 construct provided the expression of a MB3- PHO1 fusion polypeptide with an apparent molecular weight of 36.5 kDa, which was partly processed to generate mature 34 kDa MB3. About 5-10% of expressed MB3 was secreted to the yeast growth medium, and about 40-50% of the 36.5 kDa fusion polypeptide was cleaved (Table 4). *Pichia* recombinants transformed by pPIC9/MB3 or pPIC9K/MB3 constructs expressed only MB3 fused with α-factor, yielding a fusion polypeptide of approximately 45 kDa. There was no evidence of any cleavage or processing of that fusion protein.

Preliminary studies on the isolation and purification of recombinant MB3 (pHIL-D2/MB3 containing transformants) suggest that when expressed in *P.*

pastoris, MB3 may form trimers under native conditions, and that the native protein is resistant to trypsin digestion. These results are similar to those which have been observed for the wild-type counterpart.

An increase in the yield of expressed MB3 may be obtained by using strains of *Pichia* which contain multiple copies of the MB3 expression cassette. Strains harboring multiple copies exist naturally within transformed cell populations at <10% frequency. These strains may be identified either by directly screening large numbers of transformants for levels of MB3 expression via SDS-PAGE or immunoblotting, or indirectly screening by "dot blot" hybridization to select for clones containing multiple copies of the MB3 gene (Cregg *et al.*, *Bio/Technology 11*:905-910 (1993)). Alternatively, such multiple integrated clones may be constructed by using a new pAO815 vector (Invitrogen), which allows cloning of multiple copies of the gene of interest via repeated cassette insertion steps (*Ibid.* at p. 907). Scale-up procedures using a fermenter will provide higher yeast cell densities and therefore improve the yields of the expressed proteins by at least 5-10 times. Optimization of protein expression (*i.e.*, growth media composition, buffering capacity, casamino acids supplementation, increase of methanol concentration, etc.) may be carried out with routine experimentation.

Another way to identify *Pichia* transformants having multiple copies of MB3 takes advantage of the fact that the *Pichia* expression vector pPIC9K carries the kanamycin resistance gene which confers resistance to G418; in other respects, pPIC9K corresponds to pPIC9. Spontaneous generation of multiple insertion events can then be identified by the level of resistance to G418. *Pichia* transformants are selected on histidine-deficient medium and screened for their level of resistance to G418. An increased level of resistance to G418 indicates multiple copies of the kanamycin resistance gene.

Thus, the present invention relates to a method of expressing an outer membrane meningococcal group B porin protein, in particular, the class 2 and class 3 porin proteins.

In one embodiment, the present invention relates to a method of expressing the outer membrane meningococcal group B porin protein in *E. coli* comprising:

(a) transforming *E. coli* by a vector comprising a selectable marker and a gene coding for a protein selected from the group consisting of:

(i) a mature porin protein, and

(ii) a fusion protein comprising a mature porin protein fused to amino acids 1 to 20 or 22 of the T7 gene ϕ10 capsid protein;

wherein said gene is operably linked to the T7 promoter;

(b) growing the transformed *E. coli* in a culture media containing a selection agent, and

(c) inducing expression of said protein;

wherein the protein so produced comprises more than about 2% of the total protein expressed in the *E. coli*.

In a preferred embodiment, the meningococcal group B porin protein or fusion protein expressed comprises more than about 5% of the total proteins expressed in *E. coli*. In another preferred embodiment, the meningococcal group B porin protein or fusion protein expressed comprises more than about 10% of the total proteins expressed in *E. coli*. In yet another preferred embodiment, the meningococcal group B porin protein or fusion protein expressed comprises more than about 30% of the total proteins expressed in *E. coli*.

Examples of plasmids which contain the T7 inducible promotor include the expression plasmids pET-17b, pET-11a, pET-24a-d(+) and pET-9a, all of which are commercially available from Novagen (565 Science Drive, Madison, WI 53711). These plasmids comprise, in sequence, a T7 promoter, optionally a lac operator, a ribosome binding site, restriction sites to allow insertion of the structural gene and a T7 terminator sequence. *See*, the Novagen catalogue, pages 36-43 (1993).

In a preferred embodiment, *E. coli* strain BL21 (DE3) Δ*ompA* is employed. The above mentioned plasmids may be transformed into this strain or the wild-type strain BL21(DE3). *E. coli* strain BL21 (DE3) Δ*ompA* is preferred as no OmpA protein is produced by this strain which might contaminate the purified porin protein and create undesirable immunogenic side effects.

The transformed *E. coli* are grown in a medium containing a selection agent, e.g. any β-lactam to which *E. coli* is sensitive such as ampicillin. The pET expression vectors provide selectable markers which confer antibiotic resistance to the transformed organism.

High level expression of meningococcal group B porin protein can be toxic in *E. coli*. Surprisingly, the present invention allows *E. coli* to express the protein to a level of at least almost 30% and as high as >50% of the total cellular proteins.

In another embodiment, the present invention relates to a method of expressing an outer membrane meningococcal group B porin protein in yeast comprising:

(a) ligating into a plasmid having a selectable marker a gene coding for a protein selected from the group consisting of :

(i) a mature porin protein, and

(ii) a fusion protein comprising a mature porin protein fused to a yeast secretion signal peptide;

wherein said gene is operably linked to a yeast promoter;

(b) transforming the plasmid containing the gene into a yeast strain;

(c) selecting the transformed yeast by growing said yeast in a culture medium allowing selection of said transformed yeast;

(d) growing the transformed yeast, and

(e) inducing expression of said protein to give yeast containing said protein.

Transformation of the yeast host may be accomplished by any one of several techniques that are well known by those of ordinary skill in the art. These

techniques include direct or liposome-mediated transformation of yeast cells whose cell wall has been partially or completely destroyed to form spheroplasts, treatment of the host with alkali cations and PEG, and freeze-thawing combined with PEG treatment. (*See* Weber *et al.*, *Nonconventional Yeasts: Their Genetics and Biotechnological Applications, CRC Crit. Rev. Biotechnol.* 7: 281, 317 (1988) and the references cited therein, all of which are hereby fully incorporated by reference.)

In another preferred embodiment, the mature porin protein or fusion protein expressed comprises more than about 2% of the total protein expressed in the yeast host. In yet another preferred embodiment, the mature porin protein or fusion protein expressed comprises about 3-5% of the total protein expressed in the yeast host.

In another preferred embodiment, the mature porin protein is the *Neisseria meningitidis* mature outer membrane class 3 protein from serogroup B.

In another preferred embodiment, the present invention relates to performing the above method of expressing the outer membrane meningococcal group B porin protein or fusion protein in yeast, wherein said yeast is selected from the group consisting of: *Saccharomyces cerevisiae, Schizosaccharomyces pombe, Saccharomyces uvarum, Saccharomyces carlsbergensis, Saccharomyces diastaticus, Candida tropicalis, Candida maltosa, Candida parapsilosis, Pichia pastoris, Pichia farinosa, Pichia pinus, Pichia vanrijii, Pichia fermentans, Pichia guilliermondii, Pichia stipitis, Saccharomyces telluris, Candida utilis, Candida guilliermondii, Hansenula henricii, Hansenula capsulata, Hansenula polymorpha, Hansenula saturnus, Lypomyces kononenkoae, Kluyveromyces marxianus, Candida lipolytica, Saccaromycopsis fibuligera, Saccharomycodes ludwigii, Saccharomyces kluyveri, Tremella mesenterica, Zygosaccharomyces acidofaciens, Zygosaccharomyces fermentati, Yarrowia lipolytica,* and *Zygosaccharomyces soja.* Many of these yeast hosts are available from the American Type Culture Collection, Rockville, Md.

In another preferred embodiment, the nucleotide sequence of the gene encoding the mature porin protein or fusion protein incorporates codons which are optimized for yeast codon usage. In yet another preferred embodiment, the nucleotide sequence of the gene encoding the mature porin protein which has been optimized for yeast codon usage is the nucleotide sequence SEQ ID NO: 26.

In another preferred embodiment, the yeast secretion signal peptide is selected from the group consisting of the secretion signal of the *S. cerevisiae* α-mating factor prepro-peptide and the secretion signal of the *P. pastoris* acid phosphatase gene.

In another preferred embodiment, the yeast secretes the protein or fusion protein.

In another preferred embodiment, the yeast promoter to which the gene is operably linked is selected from a group consisting of the AOX1 promoter, the GAPDH promoter, the PHO5 promoter, the glyceraldehyde-3-phosphate dehydrogenase (TDH3) promoter, the ADH1 promoter, the MFα1 promoter, and the GAL10 promoter. Examples of plasmids which contain the AOX1 promoter include the expression plasmids pHIL-D2, pHIL-S1, pPIC9, and pPIC9K. These plasmids comprise, in sequence, an AOX1 promoter, restriction sites to allow insertion of the structural gene, an AOX1 transcription termination fragment, an open reading frame encoding HIS4 (histidinol dehydrogenase), an ampicillin resistance gene, and a ColE1 origin. In addition, plasmids pPIC9 and pPIC9K comprise the α-factor secretion signal of *S. cerevisiae*, and plasmid pHIL-S1 comprises the *PHO1* secretion signal of *P. pastoris*. pPIC9K also includes the kanamycin resistance gene, which confers resistance to G418 in *Pichia*. The level of G418 resistance in *Pichia* transformants can be used to identify cells which have undergone multiple insertion events. This occurs at a frequency of 1-10%. An increased level of resistance to G418 indicates the presence of multiple copies of the kanamycin resistance gene and of the gene of interest. *See* the Novagene catalogue, Version E, pp. 19-22 (1995).

In another preferred embodiment, yeast host strains having a mutation in a suitable marker gene which causes the strain to have specific nutritional requirements are employed. Expression plasmids carrying a functional copy of the mutated gene as well as a copy of the meningococcal group B porin protein or fusion protein are then transformed into the yeast host strain, and transformants are selected on the basis of their ability to grow on medium lacking the required nutrient. Examples of suitable marker genes, followed by their *S. cerevisiae* notation, include the genes encoding imidazole glycerol phosphate dehydrogenase (*HIS3*), beta-isopropylmalate dehydrogenase (*LEU2*), tryptophan synthase (*TRP5*), argininosuccinate lyase (*ARG4*), *N*-(5'-phosphorilosyl) anthranilate isomerase (*TRP1*), histidinol dehydrogenase (*HIS4*), orotidine-5-phosphate decarboxylase (*URA3*), dihydroorotate dehydrogenase (*URA1*), galactokinase (*GAL1*), and alpha-aminodipate reductase (*LYS2*). After transformed yeast host cells are selected on the basis of their ability to grow in medium lacking the appropriate nutrient, the cells are screened for integration of the meningococcal group B porin protein or fusion protein at the correct loci. This screening is performed by methods well known to those of ordinary skill in the art, for example, by selecting for transformants which grow slowly on medium which lacks the nutrient used to confirm transformation and includes methanol in order to induce expression of the outer membrane meningococcal group B porin protein or fusion protein from the AOX1 promoter. These transformants are then grown up in glycerol-containing medium, and expression of the meningococcal group B porin protein or fusion protein is then induced by the addition of methanol.

In a more preferred embodiment, *P. pastoris* host strains GS115 or KM71 are employed. These strains have a mutation in the histidinol dehydrogenase gene (*his4*) which prevents them from synthesizing histidine. The expression plasmids pHIL-D2, pHIL-S1, pPIC9, and pPIC9K carry the *HIS4* gene which complements *his4* in the host, allowing selection of transformants on histidine-deficient medium. After transformed *P. pastoris* host cells are selected in

histidine-deficient medium, the cells are screened for integration of the meningococcal group B porin protein or fusion protein at the correct loci by selecting for transformants which grow slowly on his$^-$, methanol$^+$ plates. These transformants, which become mutated at the *AOX1* locus when the MB3 gene inserts into the host genome, are only capable of slow growth on methanol, as they are metabolizing methanol with the less efficient *AOX2* gene product. The transformants are then grown up in glycerol-containing medium, and expression of the meningococcal group B porin protein or fusion protein is then induced by the addition of methanol.

In a most preferred embodiment, the present invention relates to performing the above method of expressing the outer membrane meningococcal group B porin protein in yeast, wherein said yeast is *Pichia pastoris*.

In another preferred embodiment, the present invention relates to a vaccine for inducing an immune response in an animal comprising the outer membrane meningococcal group B porin protein or fusion protein thereof, produced according to the above-described methods, together with a pharmaceutically acceptable diluent, carrier, or excipient, wherein the vaccine may be administered in an amount effective to elicit an immune response in an animal to *Neisseria meningitidis*. In a preferred embodiment, the animal is selected from the group consisting of humans, cattle, pigs, sheep, and chickens. In another preferred embodiment, the animal is a human.

In another preferred embodiment, the present invention relates to the above-described vaccine, wherein said outer membrane meningococcal group B porin protein or fusion protein thereof is conjugated to a meningococcal group B capsular polysaccharide (CP). Such capsular polysaccharides may be prepared as described in Ashton, F.E. *et al., Microbial Pathog. 6*:455-458 (1989); Jennings, H.J. *et al., J. Immunol. 134*:2651 (1985); Jennings, H.J. *et al., J. Immunol. 137*:1708-1713 (1986); Jennings, H.J. *et al., J. Immunol. 142*:3585-3591 (1989); Jennings, H.J., "Capsular Polysaccharides as Vaccine Candidates,"

in *Current Topics in Microbiology and Immunology*, *150*:105-107 (1990); the contents of each of which are fully incorporated by reference herein.

The invention also relates to a vaccine capable of simultaneously inducing an immune response against any one of several *N. meningitidis* serogroups. Thus, in another preferred embodiment, the invention relates to a trivalent vaccine comprising the capsular polysaccharides from each of three different serogroups of *N. meningitidis*. Specifically, the vaccine of the invention comprises group A meningococcal polysaccharide (GAMP), group B meningococcal polysaccharide (GBMP), and group C meningococcal polysaccharide (GCMP) antigens, together with a pharmaceutically acceptable carrier.

In a preferred embodiment, group A meningococcal polysaccharide (GAMP), group B meningococcal polysaccharide (GBMP), and group C meningococcal polysaccharide (GCMP) antigens are each conjugated to a protein carrier, thus yielding GAMP, GCMP and GBMP polysaccharide antigen conjugates.

Of course, it will be understood by those of ordinary skill that a number of carrier proteins will be suitable to be used in the polysaccharide-protein conjugates included in the vaccine of the invention. A suitable carrier protein will be one which is safe for administration to mammals, and which is immunologically effective as a carrier. Safety includes absence of primary toxicity and minimal risk of allergic complications.

In general, any heterologous protein could serve as a carrier antigen. The protein may be, for example, native toxin or detoxified toxin (also termed toxoid). In addition, genetically altered proteins which are antigenically similar to toxins and yet non-toxic may be produced by mutational techniques well-known to those of skill in the art. Such an altered toxin is termed a "cross reacting material," or CRM. CRM_{197} is noteworthy, because it differs from native diphtheria toxin at only one amino acid residue, and is immunologically indistinguishable from the native toxin (Anderson, P.W., *Infect. Immun. 39*:233-238 (1983)).

It will be understood by those of skill in the art that the polysaccharide-protein carrier conjugates of the vaccine may be produced by several different methods. The types of covalent bonds which couple a polysaccharide to a protein carrier, and the means of producing them, are well known to those of skill in the art. Details concerning the chemical means by which the two moieties can be linked may be found in U.S. Patent No. 5,371,197, and 4,902,506, the contents of which are herein incorporated by reference in their entirety. One such method is the reductive amination process described in Schwartz and Gray (*Arch. Biochim. Biophys. 181*:542-549 (1977)). This process involves reacting the reducing capsular polysaccharide fragment and bacterial toxin or toxoid in the presence of cyanoborohydride ions, or another reducing agent. Such a process will not adversely affect the toxin or toxoid or the capsular polysaccharide (U.S. Patent No. 4,902,506). Such a conjugation process is also described in Examples 12-14, below.

While tetanus and diphtheria toxins are the prime candidates for carrier proteins, owing to their history of safety, there may be overwhelming reasons, well known to those of ordinary skill in the art, to use another protein. For example, another protein may be more effective as a carrier, or production economics may be significant. Other candidates include toxins or toxoids of pseudomonas, staphylococcus, streptococcus, pertussis and entertoxigenic bacteria, including *Escherichia coli*. A preferred carrier protein to which the group B meningococcal polysaccharide may be conjugated is the class 3 porin protein (PorB) of group B *N. meningitidis*. A preferred protein carrier protein to which GAMP antigen and GCMP antigen may be conjugated is tetanus toxoid.

It is known in the art that the immunogenicity of GBMP is limited in humans, and especially in infant humans, and that direct covalent couplings of the group B polysaccharide to tetanus toxoid yielded a conjugate which failed to induce a significant polysaccharide-specific response in either rabbits (Jennings, H.J. and Lugowski, C., *J. Immunol. 127*:1011-1018 (1981)) or mice (Jennings,

H.J. *et al., J. Immunol. 137*:1708-1713 (1986)). This failure prompted interest in the direct chemical modification of the group B polysaccharide. This was done with the idea of creating synthetic epitopes capable of modulating the immune response in such a way as to produce enhanced levels of cross-reactive B polysaccharide-specific antibodies (Jennings, H.J. *et al., J. Immunol. 137*:1708-1713 (1986)).

It will be understood by those of ordinary skill in the art that in selecting possible chemical modifications of the group B polysaccharide (Jennings, H.J. *et al., J. Immunol. 137*:1708-1713 (1986)), two factors should be considered. First, the chemical modification should be able to be accomplished with facility and with the minimum of degradation of the polysaccharide. Second, in order to produce cross-reactive B polysaccharide-specific antibodies, the antigenicity of the modified polysaccharide to B polysaccharide-specific antibodies should be preserved. It will be understood by those of skill in the art that the ideal chemical modification of group B polysaccharide will retain both the carboxylate and the N-carbonyl groups (Jennings, H.J. *et al., J. Immunol. 137*:1708-1713 (1986)). The most preferred modification which satisfies the above criteria is a modification wherein the N-acetyl groups of the sialic acid residues of the B polysaccharide are removed by strong base and replaced by N-propionyl groups (see Examples 6 and 14).

In a more preferred embodiment, the N-propionylated GBMP is subsequently conjugated to a carrier protein. While any carrier protein which enhances the immunogenicity of N-propionylated GBMP may be used, a preferred protein carrier is the class 3 outer membrane protein of group B *N. meningitidis* (MB3, or PorB).

Thus, in still another preferred embodiment, GBMP antigen is conjugated to PorB after having been N-propionylated.

Preferably, the capsular polysaccharide (CP), which may be group A, B or C meningococcal polysaccharide, is isolated according to Frasch, C.E., "Production and Control of *Neisseria meningitidis* Vaccines" in *Bacterial*

Vaccines, Alan R. Liss, Inc., pages 123-145 (1990), the contents of which are fully incorporated by reference herein, as follows:

Grow organisms in modified Franz medium 10 to 20 hrs

↓ Heat kill, 55°C, 10 min

Remove inactivated cells by centrifugation

↓ Add Cetavlon to 0.1%

Precipitate CP from culture broth

↓ Add calcium chloride to 1 M

Dissolve CP then centrifuge to remove cellular debris

↓ Add ethyl alcohol to 25%

Remove precipitated nucleic acids by centrifugation

↓ Add ethyl alcohol to 80%

Precipitate crude CP and remove alcohol

The crude CP is then further purified by gel filtration chromatography after partial depolymerization with dilute acid, e.g. acetic acid, formic acid, and trifluoroacetic acid (0.01-0.5 N), to give a mixture of polysaccharides having an average molecular weight of 10,000-20,000. Where the CP is GBMP, purified GBMP is then N-deacetylated with NaOH in the presence of sodium borohydride and N-propionylated to afford N-Pr GBMP. Thus, the CP that may be employed in the conjugate vaccines of the present invention may be CP fragments, N-deacylated CP and fragments thereof, as well as N-Pr CP and fragments thereof, so long as they induce active immunity when employed as part of a CP-porin protein conjugate (see Examples 6 and 14).

In a further preferred embodiment, the present invention relates to a method of preparing a polysaccharide conjugate comprising: obtaining the above-described outer membrane meningococcal group B porin protein or fusion protein thereof; obtaining a CP from a *Neisseria meningitidis* organism; and conjugating the protein to the CP.

The conjugates of the invention may be formed by reacting the reducing end groups of the CP to primary amino groups of the porin by reductive amination. The reducing groups may be formed by selective hydrolysis or specific oxidative cleavage, or a combination of both. Preferably, the CP is conjugated to the porin protein by the method of Jennings *et al.*, U.S. Patent No. 4,356,170, the contents of which are fully incorporated by reference herein, which involves controlled oxidation of the CP with periodate followed by reductive amination with the porin protein.

The vaccine of the present invention comprises the meningococcal group B porin protein, fusion protein or conjugate vaccine, or the trivalent GAMP. GBMP and GCMP vaccine, in an amount effective depending on the route of administration. Although subcutaneous or intramuscular routes of administration are preferred, the meningococcal group B porin protein, fusion protein or vaccine of the present invention can also be administered by an intraperitoneal or intravenous route. One skilled in the art will appreciate that the amounts to be administered for any particular treatment protocol can be readily determined without undue experimentation. Suitable amounts might be expected to fall within the range of 2 micrograms of the protein per kg body weight to 100 micrograms per kg body weight.

Thus, in a preferred embodiment, the vaccine comprises about 2 μg of the GAMP, GCMP and GBMP polysaccharide antigen conjugates.

In another preferred embodiment, the vaccine comprises about 5 μg of the GAMP, GCMP and GBMP polysaccharide antigen conjugates.

In yet another referred embodiment, the vaccine comprises about 2 μg of the GAMP and GCMP polysaccharide antigen conjugates, and about 5 μg of the GBMP polysaccharide antigen conjugate.

The vaccine of the present invention may be employed in such forms as capsules, liquid solutions, suspensions or elixirs for oral administration, or sterile liquid forms such as solutions or suspensions. Any inert carrier is preferably used, such as saline, phosphate-buffered saline, or any such carrier in which the

meningococcal group B porin protein, fusion protein or conjugate vaccine have suitable solubility properties. The vaccines may be in the form of single dose preparations or in multi-dose flasks which can be used for mass vaccination programs. Reference is made to Remington's *Pharmaceutical Sciences*, Mack Publishing Co., Easton, PA, Osol (ed.) (1980); and *New Trends and Developments in Vaccines*, Voller *et al.* (eds.), University Park Press, Baltimore, MD (1978), for methods of preparing and using vaccines.

The vaccines of the present invention may further comprise adjuvants which enhance production of porin-specific antibodies. Such adjuvants include, but are not limited to, various oil formulations such as Freund's complete adjuvant (CFA), stearyl tyrosine (ST, *see* U.S. Patent No. 4,258,029), the dipeptide known as MDP, saponin, aluminum hydroxide, and lymphatic cytokine.

Freund's adjuvant is an emulsion of mineral oil and water which is mixed with the immunogenic substance. Although Freund's adjuvant is powerful, it is usually not administered to humans. Instead, the adjuvant alum (aluminum hydroxide) or ST may be used for administration to a human. The meningococcal group B porin protein or a conjugate vaccine thereof may be absorbed onto the aluminum hydroxide from which it is slowly released after injection. The meningococcal group B porin protein or group A, B and C meningococcal polysaccharide conjugate vaccine may also be encapsulated within liposomes according to Fullerton, U.S. Patent No. 4,235,877.

In another preferred embodiment, the present invention relates to a method of inducing an immune response in an animal comprising administering to the animal the vaccine of the invention, produced according to methods described, in an amount effective to induce an immune response.

In a further embodiment, the invention relates to a method of purifying the above-described outer membrane meningococcal group B porin protein or fusion protein comprising: lysing the transformed *E. coli* to release the meningococcal group B porin protein or fusion protein as part of insoluble inclusion bodies; washing the inclusion bodies with a buffer to remove

contaminating *E. coli* cellular proteins; resuspending and dissolving the inclusion bodies in an aqueous solution of a denaturant; diluting the resultant solution in a detergent; and purifying the solubilized meningococcal group B porin protein by gel filtration.

The lysing step may be carried out according to any method known to those of ordinary skill in the art, e.g. by sonication, enzyme digestion, osmotic shock, or by passing through a mull press.

The inclusion bodies may be washed with any buffer which is capable of solubilizing the *E. coli* cellular proteins without solubilizing the inclusion bodies comprising the meningococcal group B porin protein. Such buffers include but are not limited to TEN buffer (50 mM Tris HCl, 1 mM EDTA, 100 mM NaCl, pH 8.0), Tricine, Bicine and HEPES.

Denaturants which may be used in the practice of the invention include 2 to 8 M urea or about 2 to 6 M guanidine HCl, more preferably, 4 to 8 M urea or about 4 to 6 M guanidine HCl, and most preferably, about 8 M urea or about 6 M guanidine HCl.

Examples of detergents which can be used to dilute the solubilized meningococcal group B porin protein include, but are not limited to, ionic detergents such as SDS and cetavlon (Calbiochem); non-ionic detergents such as Tween, Triton X, Brij 35 and octyl glucoside; and zwitterionic detergents such as 3,14-Zwittergent, empigen BB and Champs.

Finally, the solubilized outer membrane meningococcal group B porin protein may be purified by gel filtration to separate the high and low molecular weight materials. Types of filtration gels include but are not limited to Sephacryl-300, Sepharose CL-6B, and Bio-Gel A-1.5m. The column is eluted with the buffer used to dilute the solubilized protein. The fractions containing the porin or fusion thereof may then be identified by gel electrophoresis, the fractions pooled, dialyzed, and concentrated.

Finally, substantially pure (>95%) porin protein and fusion protein may be obtained by passing the concentrated fractions through a Q sepharose high performance column.

In another embodiment, the present invention relates to expression of the meningococcal group B porin protein gene which is part of a vector which comprises the T7 promoter, which is inducible. If a promoter is an inducible promoter, then the rate of transcription increases in response to an inducing agent. The T7 promoter is inducible by the addition of isopropyl β-D-thiogalactopyranoside (IPTG) to the culture medium. Alternatively, the Tac promotor or heat shock promotor may be employed. Preferably, the meningococcal group B porin protein gene is expressed from the pET-17 expression vector or the pET-11a expression vector, both of which contain the T7 promoter.

The cloning of the meningococcal group B porin protein gene or fusion gene into an expression vector may be carried out in accordance with conventional techniques, including blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide appropriate termini, filling in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and ligation with appropriate ligases. Reference is made to Sambrook *et al., Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor, New York, Cold Spring Harbor Laboratory Press (1989), for general methods of cloning.

The meningococcal group B porin protein and fusion protein expressed according to the present invention must be properly refolded in order to achieve a structure which is immunologically characteristic of the native protein. In yet another embodiment, the present invention relates to a method of refolding the above-described outer membrane protein and fusion protein comprising: lysing the transformed cells to release the meningococcal group B porin protein or fusion protein as part of insoluble inclusion bodies; washing the inclusion bodies with a buffer to remove contaminating cellular proteins: resuspending and

dissolving the inclusion bodies in an aqueous solution of a denaturant; diluting the resultant solution in a detergent; and purifying the solubilized meningococcal group B porin protein or fusion protein by gel filtration to give the refolded protein in the eluant. Surprisingly, it has been discovered that the folded trimeric meningococcal group B class 2 and class 3 porin proteins and fusion proteins are obtained directly in the eluant from the gel filtration column.

In another preferred embodiment, the present invention relates to a substantially pure refolded outer membrane meningococcal group B porin protein and fusion protein produced according to the above-described methods. A substantially pure protein is a protein that is generally lacking in other cellular *Neisseria meningitidis* components as evidenced by, for example, electrophoresis. Such substantially pure proteins have a purity of >95%, as measured by densitometry on an electrophoretic gel after staining with Coomassie blue or silver stains.

The following examples are illustrative, but not limiting, of the method and compositions of the present invention. Other suitable modifications and adaptations of the variety of conditions and parameters normally encountered in this art which are obvious to those skilled in the art are within the spirit and scope of the present invention.

Examples

Example 1. Cloning of the Class 3 Porin Protein from Group B Neisseria meningitidis

Materials and Methods

Organisms: The Group B *Neisseria meningitidis* strain 8765 (B:15:P1,3) was obtained from Dr. Wendell Zollinger (Walter Reed Army Institute for Research) and grown on agar media previously described (Swanson, J.L., *Infect.*

Immun. 21:292-302 (1978)) in a candle extinction jar in an incubator maintained at 30°C. *Escherichia coli* strains DME558 (from the collection of S. Benson; Silhavy, T.J. *et al.*, "Experiments with Gene Fusions," Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1984), BRE51 (Bremer, E. *et al.*, *FEMS Microbiol. Lett. 33*:173-178 (1986)) and BL21(DE3) were grown on LB agar plates at 37°C.

P1 Transduction: A $P1_{vir}$ lysate of *E. coli* strain DME558 was used to transduce a tetracycline resistance marker to strain BRE51 (Bremer, E., *et al.*, *FEMS Microbiol. Lett. 33*:173-178 (1986)) in which the entire *ompA* gene had been deleted (Silhavy, T.J., *et al.*, *Experiments with Gene Fusions*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984)). Strain DME558, containing the tetracycline resistance marker in close proximity of the *ompA* gene, was grown in LB medium until it reached a density of approximately 0.6 OD at 600 nm. One tenth of a milliliter of 0.5 M $CaCl_2$ was added to the 10 ml culture and 0.1 ml of a solution containing 1 x 10^9 PFU of $P1_{vir}$. The culture was incubated for 3 hours at 37°C. After this time, the bacterial cell density was visibly reduced. 0.5 ml of chloroform was added and the phage culture stored at 4°C. Because typically 1-2% of the *E. coli* chromosome can be packaged in each phage, the number of phage generated covers the entire bacterial host chromosome, including the tetracycline resistance marker close to the *ompA* gene.

Next, strain BRE51, which lacks the *ompA* gene, was grown in LB medium overnight at 37°C. The overnight culture was diluted 1:50 into fresh LB and grown for 2 hr. The cells were removed by centrifugation and resuspended in MC salts. 0.1 ml of the bacterial cells were mixed with 0.05 of the phage lysate described above and incubated for 20 min. at room temperature. Thereafter, an equal volume of 1 M sodium citrate was added and the bacterial cells were plated out onto LB plates containing 12.5 μg/ml of tetracycline. The plates were incubated overnight at 37°C. Tetracycline resistant (12 μg/ml) transductants were screened for lack of OmpA protein expression by SDS-PAGE

and Western Blot analysis, as described below. The bacteria resistant to the antibiotic have the tetracycline resistance gene integrated into the chromosome very near where the *ompA* gene had been deleted from this strain. One particular strain was designated BRE-T^R.

A second round of phage production was then carried out with the strain BRE-T^R, using the same method as described above. Representatives of this phage population contain both the tetracycline resistance gene and the OmpA deletion. These phage were then collected and stored. These phage were then used to infect *E. coli* BL21(DE3). After infection, the bacteria contain the tetracycline resistance marker. In addition, there is a high probability that the OmpA deletion was selected on the LB plates containing tetracycline.

Colonies of bacteria which grew on the plates were grown up separately in LB medium and tested for the presence of the OmpA protein. Of those colonies selected for examination, all lacked the OmpA protein as judged by antibody reactivity on SDS-PAGE western blots.

SDS-PAGE and Western Blot: The SDS-PAGE was a variation of Laemmli's method (Laemmli, U.K., *Nature 227*:680-685 (1970)) as described previously (Blake and Gotschlich, *J. Exp. Med. 159*:452-462 (1984)). Electrophoretic transfer to Immobilon P (Millipore Corp. Bedford, MA) was performed according to the methods of Towbin *et al.* (Towbin, H., *et al.*, *Proc. Natl. Acad. Sci. USA 76*:4350-4354 (1979)) with the exception that the paper was first wetted in methanol. The Western blots were probed with phosphatase conjugated reagents (Blake, M.S., *et al.*, *Analyt. Biochem. 136*:175-179 (1984)).

Polymerase Chain Reaction: The method described by Feavers *et al.* (Feavers, I.M., *et al.*, *Infect. Immun. 60*:3620-3629 (1992)) was used to amplify the gene encoding the PorB. The primers selected were primers 33 (GGG GTA GAT CTG CAG GTT ACC TTG TAC GGT ACA ATT AAA GCA GGC GT) and 34 (GGG GGG GTG ACC CTC GAG TTA GAA TTT GTG ACG CAG ACC AAC) as previously described (Feavers, I.M., *et al.*, *Infect. Immun. 60*:3620-3629 (1992)). Briefly, the reaction components were as follows:

Meningococcal strain 8765 chromosomal DNA (100 ng/μl), 1 μl; 5′ and 3′ primers (1 μM) 2 μl each; dNTP (10 mM stocks), 4 μl each; 10 X PCR reaction buffer (100 mM Tris HCl, 500 mM KCl, pH 8.3), 10 μl; 25 mM $MgCl_2$, 6 μl; double distilled H_20, 62 μl; and Taq polymerase (Cetus Corp., 5 u/μl), 1 μl. The reaction was carried out in a GTC-2 Genetic Thermocycler (Precision Inst. Inc, Chicago, IL) connected to a Lauda 4/K methanol/water cooling system (Brinkman Instruments, Inc., Westbury, NY) set at 0°C. The thermocycler was programmed to cycle 30 times through: 94°C, 2 min.; 40°C, 2 min.; and 72°C, 3 min. At the end of these 30 cycles, the reaction was extended at 72°C for 3 min and finally held at 4°C until readied for analysis on a 1% agarose gel in TAE buffer as described by Maniatis (Maniatis, T., *et al.*, *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982)).

Subcloning of the PCR product: The pET-17b plasmid (Novagen, Inc.) was used for subcloning and was prepared by double digesting the plasmid with the restriction endonucleases *Bam*HI and *Xho*I (New England Biolabs, Inc., Beverly, MA). The digested ends were then dephosphorylated with calf intestinal alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN). The digested plasmid was then analyzed on a 1% agarose gel, the cut plasmid removed, and purified using the GeneClean kit (Bio101, La Jolla, CA). The PCR product was prepared by extraction with phenol-chloroform, chloroform, and finally purified using the GeneClean Kit (Bio101). The PCR product was digested with restriction endonucleases *Bgl*II and *Xho*I (New England Biolabs, Inc.). The DNA was then extracted with phenol-chloroform, precipitated by adding 0.1 volumes of 3 M sodium acetate, 5 μl glycogen (20 μg/μl), and 2.5 volumes of ethanol. After washing the DNA with 70% ethanol (vol/vol), it was redissolved in TE buffer. The digested PCR product was ligated to the double digested pET-17b plasmid described above using the standard T4 ligase procedure at 16°C overnight (*Current Protocols in Molecular Biology*, John Wiley & Sons, New York (1993)). The ligation product was then transformed into the BL21 (DE3)-

Δ*omp*A described above which were made competent by the method of Chung *et al.* (Chung, C.T., *et al.*, *Proc. Natl. Acad. Sci. USA 86*:2172-2175 (1989)). The transformants were selected on LB plates containing 50 μg/ml carbenicillin and 12μg/ml tetracycline. Several transformants were selected, cultured in LB both containing carbenicillin and tetracycline for 6 hours at 30°C. and plasmid gene expression inducted by the addition of IPTG. The temperature was raised to 37°C and the cultures continued for an additional 2 hrs. The cells of each culture were collected by centrifugation, whole cell lysates prepared, and analyzed by SDS-PAGE and Western Blot using a monoclonal antibody (4D11) which reacts with all neisserial porins.

Nucleotide Sequence Analysis: The nucleotide sequences of the cloned Class 3 porin gene DNA were determined by the dideoxy method using denatured double-stranded plasmid DNA as the template as described (*Current Protocols in Molecular Biology*, John Wiley & Sons, New York (1993)). Sequenase II kits (United States Biochemical Corp., Cleveland, OH) were used in accordance with the manufacturer's instructions. The three synthesized oligonucleotide primers (Operon Technologies, Inc., Alameda, CA) were used for these reactions. One for the 5′ end, which consisted of 5′TCAAGCTTGGTACCGAGCTC and two for the 3′ end, 5′TTTGTTAGCAGCCGGATCTG and 5′ CTCAAGACCCGTTTAGAGGCC. Overlapping, nested deletions were made by linearizing the plasmid DNA by restriction endonuclease *Bpu*1102I and the ends blunted by the addition of Thio-dNTP and Klenow polymerase (*Current Protocols in Molecular Biology*, John Wiley & Sons, New York (1993)). The linearized plasmid was then cleaved with restriction endonuclease *Xho*I and the exoII/Mung bean nuclease deletion kit used to make 3′ deletions of the plasmid (Stratagene, Inc., La Jolla, CA) as instructed by the supplier. A map of this strategy is shown in Figure 1.

Expression and purification of the PorB gene product: Using a sterile micropipette tip, a single colony of the BL21 (DE3)-Δ*omp*A containing the PorB-pET-17b plasmid was selected and inoculated into 10 ml of LB broth containing

50 µg/ml carbenicillin. The culture was incubated overnight at 30°C while shaking. The 10 ml overnight culture was then sterilely added to 1 liter of LB broth with the same concentration of carbenicillin, and the culture continued in a shaking incubator at 37°C until the OD_{600} reached 0.6-1.0. Three mls of a stock solution of IPTG (100 mM) was added to the culture and the culture incubated for an additional 30 min. Rifampicin was then added (5.88 ml of a stock solution; 34 mg/ml in methanol) and the culture continued for an additional 2 hrs. The cells were harvested by centrifugation at 10,000 rpm in a GS3 rotor for 10 min and weighed. The cells were thoroughly resuspended in 3 ml of TEN buffer (50 mM Tris HCl, 1 mM Tris HCl, 1 mM EDTA, 100 mM NaCl, pH 8.0) per gram wet weight of cells. To this was added 8 µl of PMSF stock solution (50 mM in anhydrous ethanol) and 80 µl of a lysozyme stock solution (10 mg/ml in water) per gram wet weight of cells. This mixture was stirred at room temperature for 20 min. While stirring, 4 mg per gram wet weight of cells of deoxycholate was added. The mixture was placed in a 37°C water bath and stirred with a glass rod. When the mixture became viscous, 20 µl of DNase I stock solution (1 mg/ml) was added per gram weight wet cells. The mixture was then removed from the water bath and left at room temperature until the solution was no longer viscous. The mixture was then centrifuged at 15,000 rpm in a SS-34 rotor for 20 min at 4°C. The pellet was retained and thoroughly washed twice with TEN buffer. The pellet was then resuspended in freshly prepared TEN buffer containing 0.1 mM PMSF and 8 M urea and sonicated in a bath sonicator (Heat Systems, Inc., Plain view, NY). The protein concentration was determined using a BCA kit (Pierce, Rockville, IL) and the protein concentration adjusted to less than 10 mg/ml using the TEN-urea buffer. The sample was then diluted 1:1 with 10% (weight/vol) Zwittergent 3,14 (Calbiochem, La Jolla, CA), sonicated, and loaded onto a Sephacryl S-300 molecular sieve column. The Sephacryl S-300 column (2.5 cm x 200 cm) had previously equilibrated with 100 mM Tris HCl, 200 mM NaCl, 10 mM EDTA, 0.05% Zwittergent 3,14, and 0.02% azide, pH 8.0. The column flow rate was adjusted to 8 ml/hr and 10 ml fractions were collected. The

OD_{280} of each fraction was measured and SDS-PAGE analysis performed on protein containing fractions.

Inhibition ELISA Assays: Microtiter plates (Nunc-Immuno Plate IIF, Nunc, Inc., Naperville, IL) were sensitized by adding 0.1 ml per well of porB (2 µg/ml) purified from the wild type strain 8765, in 0.1 M carbonate buffer, pH 9.6 with 0.02% azide. The plates were incubated overnight at room temperature. The plates were washed five times with 0.9% NaCl, 0.05% Brij 35, 10 mM sodium acetate pH 7.0, 0.02% azide. Human immune sera raised against the Type 15 Class 3 PorB protein was obtained from Dr. Phillip O. Livingston, Memorial-Sloan Kettering Cancer Center, New York, N.Y. The human immune sera was diluted in PBS with 0.5% Brij 35 and added to the plate and incubated for 2 hr at room temperature. The plates were again washed as before and the secondary antibody, alkaline phosphatase conjugated goat anti-human IgG (Tago Inc., Burlingame, CA), was diluted in PBS-Brij, added to the plates and incubated for 1 hr at room temperature. The plates were washed as before and *p*-nitrophenyl phosphate (Sigma Phosphatase Substrate 104) (1 mg/ml) in 0.1 diethanolamine, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 0.02% azide, pH 9.8, was added. The plates were incubated at 37°C for 1 h and the absorbance at 405 nm determined using an Elida-5 microtiter plate reader (Physica, New York, NY). Control wells lacked either the primary and/or secondary antibody. This was done to obtain a titer for each human serum which would give a half-maximal reading in the ELISA assay. This titer for each human serum would be used in the inhibition ELISA. The ELISA microtiter plate would be sensitized with purified wild type PorB protein and washed as before. In a separate V-96 polypropylene microtiter plate (Nunc, Inc.), varying amounts of either purified wild type PorB protein or the purified recombinant PorB protein were added in a total volume of 75 µl. The human sera were diluted in PBS-Brij solution to twice their half maximal titer and 75 µl added to each of the wells containing the PorB or recombinant PorB proteins. This plate was incubated for 2 hr at room temperature and centrifuged in a Sorvall RT6000 refrigerated centrifuge,

equipped with microtiter plate carriers (Wilmington, DE) at 3000 rpm for 10 min. Avoiding the V-bottom, 100 µl from each well was removed and transferred to the sensitized and washed ELISA microtiter plate. The ELISA plates are incubated for an additional 2 hr, washed, and the conjugated second antibody added as before. The plate is then processed and read as described. The percentage of inhibition is then processed and read as described. The percentage of inhibition is calculated as follows:

$$\frac{1 - (ELISA\ value\ with\ either\ PorB\ or\ rPorB\ protein\ added)}{(ELISA\ value\ without\ the\ porB\ added)} \times 100$$

Results

Polymerase Chain Reaction and Subcloning: A method to easily clone, genetically manipulate, and eventually obtain enough pure porin protein from any number of different neisserial porin genes for further antigenic and biophysical characterization has been developed. The first step toward this goal was cloning the porin gene from a Neisseria. Using a technique originally described by Feavers, *et al.* (Feavers, I.M., *et al.*, *Infect. Immun. 60*:3620-3629 (1992)), the DNA sequence of the mature porin protein from a class 3, serotype 15 porin was amplified using the chromosome of meningococcal strain 8765 as a template for the PCR reaction. Appropriate endonuclease restriction sites had been synthesized onto the ends of the oligonucleotide primers, such that when cleaved, the amplified mature porin sequence could be directly ligated and cloned into the chosen expression plasmid. After 30 cycles, the PCR products shown in Figure 2 were obtained. The major product migrated between 900bp and 1000bp which was in accord with the previous study (Feavers, I.M., *et al.*, *Infect. Immun. 60*:3620-3629 (1992)). However, a higher molecular weight product was not

seen, even though the PCR was conducted under low annealing stringencies (40°C; 50 mM KCl).

To be able to produce large amounts of the cloned porin protein, the tightly controlled expression system of Studier, *et al.* (Studier and Moffatt, *J. Mol. Biol. 189*:113-130 (1986)) was employed, which is commercially available through Novagen Inc. The amplified PCR product was cloned into the *Bam*HI-*Xho*I site of plasmid pET-17b. This strategy places the DNA sequence for the mature porin protein in frame directly behind the T7 promoter, the DNA sequence encoding for the 9 amino acid leader sequence and 11 amino acids of the mature ϕ10 protein. The Studier *E. coli* strain BL21 lysogenic for the DE3 lambda derivative (Studier and Moffatt, *J. Mol. Biol. 189*:113-130 (1986)) was selected as the expression host for the pET-17b plasmid containing the porin gene. But because it was thought that the OmpA protein, originating from the *E. coli* expression host, might tend to co-purify with the expressed meningococcal porin protein, a modification of this strain was made by P1 transduction which eliminated the *ompA* gene from this strain. Thus, after restriction endonuclease digestion of both the PCR product and the pET-17b vector and ligation, the product was transformed into BL21(DE3)-Δ*ompA* and transformants selected for ampicillin and tetracycline resistance. The restriction map of pET-17b is shown in Figure 11A, while the nucleotide sequence between the *Bgl*II and *Xho*I sites of pET-17b is shown in Figure 11B. Of the numerous colonies observed on the selection plate, 10 were picked for further characterization. All ten expressed large amounts of a protein, which migrated at the approximate molecular weight of the PorB protein, when grown to log phase and induced with IPTG. The whole cell lysate of one such culture is shown in Figure 3a. The western blot analysis with the 4D11 monoclonal antibody further suggested that the protein being expressed was the PorB protein (Figure 3b). As opposed to other studies, when neisserial porins have been cloned and expressed in *E. coli*, the host bacterial cells showed no signs of any toxic or lethal effects even after the addition of the IPTG.

The *E. coli* cells appeared viable and could be recultured at any time throughout the expression phase.

Nucleotide sequence analysis: The amount of PorB expressed in these experiments was significantly greater than that previously observed and there appeared to be no adverse effects of this expression on the host *E. coli*. To be certain that no PCR artifacts had been introduced into the meningococcal porin gene to allow for such high expression, the entire ϕ10 porin fusion was sequenced by double stranded primer extension from the plasmid. The results are shown in Figure 4. The nucleotide sequence was identical with another meningococcal serotype 15 *Por*B gene sequence previously reported by Heckels, *et al.* (Ward, M.J., *et al.*, *FEMS Microbiol. Lett. 73*:283-289 (1992)) with two exceptions which are shown. These two nucleotide differences each occur in the third position of the codon and would not alter the amino acid sequence of the expressed protein. Thus, from the nucleotide sequence, there did not appear to be any PCR artifact or mutation which might account for the high protein expression and lack of toxicity within the *E. coli*. Furthermore, this data would suggest that a true PorB protein was being produced.

Purification of the expressed porB gene product: The PorB protein expressed in the *E. coli* was insoluble in TEN buffer which suggested that when expressed, the PorB protein formed into inclusion bodies. However, washing of the insoluble PorB protein with TEN buffer removed most of the contaminating *E. coli* proteins. The PorB protein could then be solubilized in freshly prepared 8M urea and diluted into the Zwittergent 3,14 detergent. The final purification was accomplished, using a Sephacryl S-300 molecular sieve column which not only removed the urea but also the remaining contaminating proteins. The majority of the PorB protein eluted from the column having the apparent molecular weight of trimers much like the wild type PorB. The comparative elution patterns of both the wild type and the PorB expressed in the *E. coli* are shown in Figure 5. It is important to note that when the PorB protein concentration in the 8 M urea was in excess of 10 mg/ml prior to dilution into the

Zwittergent detergent, the relative amounts of PorB protein found as trimers decreased and appeared as aggregates eluting at the void volume. However, at protein concentrations below 10 mg/ml in the urea buffer, the majority of the PorB eluted in the exact same fraction as did the wild type PorB. It was also determined using a T7-Tag monoclonal antibody and western blot analysis that the 11 amino acids of the mature T7 capsid protein were retained as the amino terminus. The total yield of the meningococcal porin protein from one liter of *E. coli* was approximately 50 mg.

Inhibition ELISA Assays. In order to determine if the purified trimeric recombinant PorB had a similar antigenic conformation as compared to the PorB produced in the wild type meningococcal strain 8765, the sera from six patients which had been vaccinated with the wild type meningococcal Type 15 PorB protein were used in inhibition ELISA assays. In the inhibition assay, antibodies reactive to the native PorB were competitively inhibited with various amounts of either the purified recombinant PorB or the homologous purified wild type PorB. The results of the inhibition with the homologous purified PorB of each of the six human sera and the mean inhibition of these sera are shown in Figure 6. The corresponding inhibition of these sera with the purified recombinant PorB is seen in Figure 6B. A comparison of the mean inhibition from Figure 6 and 7 are plotted in Figure 8. These data would suggest that the antibodies contained in the sera of these six patients found similar epitopes on both the homologous purified wild type PorB and the purified recombinant PorB. This gave further evidence that the recombinant PorB had regained most if not all of the native conformation found in the wild type PorB.

Example 2. Cloning of the Class 2 Porin from Group B Neisseria Meningitidis strain BNCV M986

Genomic DNA was isolated from approximately 0.5g of Group B *Neisseria meningitidis* strain BNCV M986 (serotype 2a) using previously described methods (Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor, New York, Cold Spring Harbor Laboratory Press (1989)). This DNA then served as the template for two class 2 porin specific oligonucleotides in a standard PCR reaction. These oligonucleotides were designed to be complementary to the 5′ and 3′ flanking regions of the class 2 porin and to contain *Eco*RI restriction sites to facilitate the cloning of the fragment. The sequences of the oligonucleotides were as follows:

5′ AGC GGC TTG *GAA TTC* CCG GCT GGC TTA AAT TTC 3′ and

5′ CAA ACG AAT *GAA TTC* AAA TAA AAA AGC CTG 3′.

The polymerase chain reaction was then utilized to obtain the class 2 porin. The reaction conditions were as follows: BNCV M986 genomic DNA 200ng, the two oligonucleotide primers described above at 1 μM of each, 200 μM of each dNTP, PCR reaction buffer (10 mM Tris HCl, 50 mM KCl, pH 8.3), 1.5 mM $MgCl_2$, and 2.5 units of *Taq* polymerase, made up to 100 μl with distilled H_2O. This reaction mixture was then subjected to 25 cycles of 95°C for 1 min, 50°C for 2 min and 72°C for 1.5 min. At the end of the cycling period, the reaction mixture was loaded on a 1% agarose gel and the material was electrophoresed for 2h after which the band at 1.3 kb was removed and the DNA recovered using the Gene Clean kit (Bio 101). This DNA was then digested with *Eco*RI, repurified and ligated to *Eco*RI digested pUC19 using T_4 DNA ligase. The ligation mixture was used to transform competent *E. coli* DH5α. Recombinant plasmids were selected and sequenced. The insert was found to have a DNA sequence consistent with that of a class 2 porin. *See*, Murakami, K. *et al.*, *Infect. Immun.* *57*:2318-2323 (1989).

The plasmid pET-17b (Novagen) was used to express the class 2 porin. As described below, two plasmids were constructed that yielded two different proteins. One plasmid was designed to produce a mature class 2 porin while the other was designed to yield a class 2 porin fused to 20 amino acids from the T7 gene ϕ10 capsid protein.

Construction of the mature class 2 porin

The mature class 2 porin was constructed by amplifying the pUC19-class 2 porin construct using the oligonucleotides: 5′-CCT GTT GCA GCA *CAT ATG* GAC GTT ACC TTG TAC GGT ACA ATT AAA GC-3′ and 5 ′-CGA CAG GCT TTT TCT CGA GAC CAA TCT TTT CAG -3′. This strategy allowed the cloning of the amplified class 2 porin into the *Nde*I and *Xho*I sites of the plasmid pET-17b thus producing a mature class 2 porin. Standard PCR was conducted using the pUC19-class 2 as the template and the two oligonucleotides described above. This PCR reaction yielded a 1.1kb product when analyzed on a 1.0% agarose gel. The DNA obtained from the PCR reaction was gel purified and digested with the restriction enzymes *Nde*I and *Xho*I. The 1.1kb DNA produced was again gel purified and ligated to *Nde*I and *Xho*I digested pET-17b using T_4 DNA ligase. This ligation mixture was then used to transform competent *E. coli* DH5α. Colonies that contained the 1.1kb insert were chosen for further analysis. The DNA from the DH5α clones was analyzed by restriction mapping and the cloning junctions of the chosen plasmids were sequenced. After this analysis, the DNA obtained from the DH5α clones was used to transform *E. coli* BL21(DE3)-Δ*ompA*. The transformants were selected to LB-agar containing 100 μg/ml of carbenicillin. Several transformants were screened for their ability to make the class 2 porin protein. This was done by growing the clones in LB liquid medium containing 100 μg/ml of carbenicillin and 0.4% glucose at 30°C to OD_{600} = 0.6 then inducing the cultures with IPTG (0.4 mM). The cells were then disrupted and the cell extract was analyzed by SDS-PAGE. The nucleotide sequence and

translated amino acid sequence of the mature class II porin gene cloned into pET-17b are shown in Figures 9A and 9B.

Construction of the fusion class 2 porin

The fusion class 2 porin was constructed by amplifying the pUC19-class 2 porin construct using the oligonucleotides: 5'-CCT GTT GCA GCG GAT CCA GAC GTT ACC TTG TAC GGT ACA ATT AAA GC- 3' and 5'-CGA CAG GCT TTT TCT CGA GAC CAA TCT TTT CAG -3'. This strategy allowed the cloning of the amplified class 2 porin into the *Bam*HI and *Xho*I sites of the plasmid pET-17b thus producing a fusion class 2 porin containing an additional 22 amino acids at the N-terminus derived from the T7 ϕ10 capsid protein contained in the plasmid. Standard PCR was conducted using the pUC19-class 2 as the template and the two oligonucleotides described above. The PCR reaction yielded a 1.1kb product when analyzed on a 1.0% agarose gel. The DNA obtained from the PCR reaction was gel purified and digested with the reaction enzymes *Bam*HI and *Xho*I. The 1.1kb product produced was again gel purified and ligated to *Bam*HI and *Xho*I digested pET-17b using T_4 DNA ligase. This ligation mixture was then used to transform competent *E. coli* DH5α. Colonies that contained the 1.1kb insert were chosen for further analysis. The DNA from the DH5α clones was analyzed by restriction enzyme mapping and the cloning junctions of the chosen plasmids were sequenced. The nucleotide sequence and translated amino acid sequence of the fusion class II porin gene cloned into the expression plasmid pET-17b are shown in Figures 10A and 10B. After this analysis, the DNA obtained from the DH5α clones was used to transform *E. coli* BL21(DE3)-Δ*ompA*. The transformants were selected on LB-agar containing 100 μg/ml of carbenicillin. Several transformants were screened for their ability to make the class 2 porin protein. This was done by growing the clones in LB liquid medium containing 100 μg/ml of carbenicillin and 0.4% glucose at 30°C to OD_{600}

= 0.6 then inducing the cultures with IPTG (0.4 mM). The cells were then disrupted and the cell extract was analyzed by SDS-PAGE.

Example 3. Cloning and Expression of the Mature class 3 porin from Group B Neisseria meningitidis strain 8765 in E. coli

Genomic DNA was isolated from approximately 0.5 g of Group B *Neisseria meningitidis* strain 8765 using the method described above (Sambrook *et al., Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor, New York, Cold Spring Harbor Laboratory Press (1989)). This DNA then served as the template for two class 3 porin specific oligonucleotides in a standard PCR reaction.

The mature class 3 porin was constructed by amplifying the genomic DNA from 8765 using the oligonucleotides: 5'-GTT GCA GCA *CAT ATG* GAC GTT ACC CTG TAC GGC ACC-3' and 5'-GGG GGG ATG GAT CCA GAT TAG AAT TTG TGG CGC AGA CCG ACA CC-3'. This strategy allowed the cloning of the amplified class 3 porin into the *Nde*I and *BamH*I sites of the plasmid pET-24a+ (Figures 13A and 13B), thus producing a mature class 3 porin. Standard PCR was conducted using the genomic DNA isolated from 8765 as the template and the two oligonucleotides described above.

The reaction conditions were as follows: 8765 genomic DNA 200 ng, the two oligonucleotide primers described above at 1 μM of each, 200 μM of each dNTP, PCR reaction buffer (10 mM Tris HCl, 50 mM KCl, pH 8.3), 1.5 mM $MgCl_2$, and 2.5 units of *Taq* polymerase, and made up to 100 μl with distilled water. This reaction mixture was then subjected to 25 cycles of 95°C for 1 min, 50°C for 2 min and 72°C for 1.5 min.

This PCR reaction yielded about 930 bp of product, as analyzed on a 1% agarose gel. The DNA obtained from the PCR reaction was gel purified and digested with the restriction enzymes *Nde*I and *Bam*HI. The 930 bp product was

again gel purified and ligated to *Nde*I and *Bam*HI digested pET-24a(+) using T4 ligase. This ligation mixture was then used to transform competent *E. coli* DH5α. Colonies that contained the 930 bp insert were chosen for further analysis. The DNA from the *E. coli* DH5α clones was analyzed by restriction enzyme mapping and cloning junctions of the chosen plasmids were sequenced. After this analysis, the DNA obtained from the *E. coli* DH5α clones was used to transform *E. coli* BL21(DE3)-Δ*ompA*. The transformants were selected on LB-agar containing 50 μg/ml of kanamycin. Several transformants were screened for their ability to make the class 3 porin protein. This was done by growing the clones in LB liquid medium containing 50 μg/ml of kanamycin and 0.4% of glucose at 30°C to OD_{600} = 0.6 then inducing the cultures with IPTG (1 mM). The cells were then disrupted and the cell extract was analyzed by SDS-PAGE.

Example 4. Purification and refolding of recombinant class 2 porin

E coli strain BL21(DE3)Δ*omp*A [pNV-5] is grown to mid-log phase (OD = 0.6 at 600 nm) in Luria broth at 30°C. IPTG is then added (0.4 mM final) and the cells grown an additional two hours at 37°C. The cells were then harvested and washed with several volumes of TEN buffer (50 mM Tris-HCl, 0.2 M NaCl, 10 mM EDTA, pH = 8.0) and the cell paste stored frozen at -75°C.

For purification preweighed cells are thawed and suspended in TEN buffer at a 1:15 ratio (g/v). The suspension is passed through a Stansted cell disrupter (Stansted fluid power Ltd.) twice at 8.000 psi. The resultant solution is then centrifuged at 13,000 rpm for 20 min and the supernatant discarded. The pellet is then twice suspended in TEN buffer containing 0.5% deoxycholate and the supernatants discarded. The pellet is then suspended in TEN buffer containing 8 M deionized urea (electrophoresis grade) and 0.1 mM PMSF (3 g/10ml). The suspension is sonicated for 10 min or until an even suspension is achieved. 10 ml of a 10% aqueous solution of 3,14-zwittergen (Calbiochem) is

added and the solution thoroughly mixed. The solution is again sonicated for 10 min. Any residual insoluble material is removed by centrifugation. The protein concentration is determined and the protein concentration adjusted to 2 mg/ml with 8 M urea-10% zwittergen buffer (1:1 ratio).

This mixture is then applied to a 2.6 x 100 cm column of Sephacryl S-300 equilibrated in 100 mM Tris-HCl, 1 M NaCl, 10 mM EDTA, 20 mM $CaCl_2$, 0.05% 3,14-zwittergen, 0.02% sodium azide, pH = 8.0. The flow rate is maintained at 1 ml/min. Fractions of 10 ml are collected. The porin refolds into trimer during the gel filtration. The OD = 280 nm of each fraction is measured and those fractions containing protein are subjected to SDS gel electrophoresis assay for porin. Those fractions containing porin are pooled. The pooled fractions are either dialyzed or diluted 1:10 in 50 mM Tris HCl pH = 8.0, 0.05% 3,14-zwittergen, 5 mM EDTA, 0.1 M NaCl. The resulting solution is then applied to a 2.6 x 10 cm Q sepharose high performance column (Pharmacia) equilibrated in the same buffer. The porin is eluted with a linear gradient of 0.1 to 1 M NaCl.

Example 5. Purification and refolding of recombinant class 3 porin

E coli strain BL21 (DE3) Δ*omp*A containing the porB-pET-17b plasmid is grown to mid-log phase (OD = 0.6 at 600 nm) in Luria broth at 30°C. IPTG is then added (0.4 mM final) and the cells grown an additional two hours at 37°C. The cells were then harvested and washed with several volumes of TEN buffer (50 mM Tris-HCl, 0.2 M NaCl, 10 mM EDTA, pH = 8.0) and the cell paste stored frozen at -75°C.

For purification about 3 grams of cells are thawed and suspended in 9 ml of TEN buffer. Lysozyme is added (Sigma, 0.25 mg/ml) deoxycholate (Sigma, 1.3 mg/ml) plus PMSF (Sigma, µg/ml) and the mixture gently shaken for one hour at room temperature. During this time, the cells lyse and the released DNA

causes the solution to become very viscous. DNase is then added (Sigma, 2 μg/ml) and the solution again mixed for one hour at room temperature. The mixture is then centrifuged at 15K rpm in a S-600 rotor for 30 minutes and the supernatant discarded. The pellet is then twice suspended in 10 ml of TEN buffer and the supernatants discarded. The pellet is then suspended in 10 ml of 8 M urea (Pierce) in TEN buffer. The mixture is gently stirred to break up any clumps. The suspension is sonicated for 20 minutes or until an even suspension is achieved. 10 ml of a 10% aqueous solution of 3,14-zwittergen (Calbiochem) is added and the solution thoroughly mixed. The solution is again sonicated for 10 minutes. Any residual insoluble material is removed by centrifugation. The protein concentration is determined and the protein concentration adjusted to 2 mg/ml with 8 M urea-10% zwittergen buffer (1:1 ratio).

This mixture is then applied to a 180 x 2.5 cm column of Sephacryl S-300 (Pharmacia) equilibrated in 100 mM Tris-HCl, 1 M NaCl, 10 mM EDTA, 20 mM $CaCl_2$, 0.05% 3,14-zwittergen, pH = 8.0. The flow rate is maintained at 1 ml/min. Fractions of 10 ml are collected. The porin refolds into trimer during the gel filtration. The OD_{280} nm of each fraction is measured and those fractions containing protein are subjected to SDS gel electrophoresis assay for porin. Those fractions containing porin are pooled.

The pooled fractions are dialyzed and concentrated 4-6 fold using Amicon concentrator with a PM 10 membrane against buffer containing 100 mM Tris-HCl, 0.1 M NaCl, 10 mM EDTA, 0.05% 3,14-zwittergen, pH = 8.0. Alternatively, the pooled fractions are precipitated with 80% ethanol and resuspended with the above-mentioned buffer. Six to 10 mg of the material is then applied to a monoQ 10/10 column (Pharmacia) equilibrated in the same buffer. The porin is eluted from a shallow 0.1 to 0.6 M NaCl gradient with a 1.2% increase per min over a 50 min period. The Flow rate is 1 ml/min. The peak containing porin is collected and dialyzed against TEN buffer and 0.05% 3,14-zwittergen. The porin may be purified further by another S-300 chromatography.

Example 6. Purification and chemical modification of the polysaccharides

The capsular polysaccharide from both group B *Neisseria meningitidis* and *E. coli* K1 consists of α(2→8) polysialic acid (commonly referred to as GBMP or K1 polysaccharide). High molecular weight polysaccharide isolated from growth medium by precipitation (*see*, Frasch, C.E., "Production and Control of *Neisseria meningitidis* Vaccines" in *Bacterial Vaccines*, Alan R. Liss, Inc., pages 123-145 (1990)) was purified and chemically modified before being coupled to the porin protein. The high molecular weight polysaccharide was partially depolymerized with 0.1 M acetic acid (7 mg polysaccharide/ml), pH = 6.0 to 6.5 (70°C, 3 hrs) to provide polysaccharide having an average molecular weight of 12,000-16,000. After purification by gel filtration column chromatography (Superdex 200 prep grade, Pharmacia), the polysaccharide was N-deacetylated in the presence of $NaBH_4$ and then N-propionylated as described by Jennings *et al.* (*J. Immunol. 137*:1808 (1986)) to afford N-Pr GBMP (see Example 14). Treatment with $NaIO_4$ followed by gel filtration column purification gave the oxidized N-Pr GBMP having an average molecular weight of 12,000 daltons.

Example 7. Coupling of oxidized N-Pr GBMP to the group B meningococcal class 3 porin protein (PP)

The oxidized N-Pr GBMP (9.5 mg) was added to purified class 3 porin protein (3.4 mg) dissolved in 0.21 ml of 0.2 M phosphate buffer pH 7.5 which also contained 10% octyl glucoside. After the polysaccharide was dissolved, sodium cyanoborohydride (7 mg) was added and the reaction solution was incubated at 37°C for 4 days. The reaction mixture was diluted with 0.15 M sodium chloride solution containing 0.01% thimerosal and separated by gel filtration column chromatography using Superdex 200 PG. The conjugate (N-Pr

GBMP-PP) was obtained as single peak eluting near the void volume. Analysis of the conjugate solution for sialic acid and protein showed that the conjugate consists of 43% polysaccharide by weight. The porin protein was recovered in the conjugate in 44% yield and the polysaccharide in 12% yield. The protein recoveries in different experiments generally occur in the 50-80% range and those of the polysaccharide in the 9-13% range (see also Example 14).

Example 8. Immunogenicity studies

The immunogenicities of the N-Pr GBMP-PP conjugate and those of the N-Pr GBMP-Tetanus toxoid (N-Pr GBMP-TT) conjugate which was prepared by a similar coupling procedure were assayed in 4-6 week old outbread Swiss Webster CFW female mice. The polysaccharide (2 μg)-conjugate was administered on days 1, 14 and 28, and the sera collected on day 38. The conjugates were administered as saline solutions, adsorbed on aluminum hydroxide, or admixed with stearyl tyrosine. The sera ELISA titers against the polysaccharide antigen and bactericidal titers against *N. meningitidis* group B are summarized in Table 1.

Example 9. Expression of group B Neisseria meningitidis Outer Membrane (MB3) Using Yeast Pichia pastoris Expression System

Materials and Methods

Strains and Plasmids

Pichia pastoris GS 115 (provided by Invitrogen) has a defect in the histidinol dehydrogenase gene (his4) which prevents it from synthesizing histidine. All expression plasmids carry the HIS4 gene which complements his4

in the host, so transformants are selected for their ability to grow on histidine-deficient medium. Until transformed, GS 115 will not grow on minimal medium alone.

Expression vectors

Four different expression vectors were used that include the strong, highly-inducible AOX1 promoter for expression of foreign protein (*Pichia* Expression Kit, Invitrogen). One vector, pHIL-D2, is used for intracellular expression, while the other three (pHIL-S1, pPIC9, and pPIC9K) are used for secreted expression. Maps of the pHIL-D2, pHIL-S1, and pPIC9 vectors may be found on pp. 19-22 of the Invitrogen Instruction Manual for the *Pichia* Expression Kit, Version E, the contents of which is hereby incorporated by reference. Secretion requires the presence of a signal sequence on the expressed protein to target it to the secretory pathway. To improve the chances for success, two different kinds of vectors are included in the kit. The vector pHIL-S1 carries a native *Pichia pastoris* signal from the acid phosphatase gene, PHO1. The vectors, pPIC9 and pPIC9K (with corrected HIS4 region), both carry the secretion signal from the *S. cerevisiae* α-mating factor pre-pro peptide. The advantage of expressing secreted proteins is that *P. pastoris* secretes very low levels of native proteins. Thus, the secreted heterologous protein comprises the vast majority of the total protein in the media and serves as the first step in purification of the protein (Barr *et al.*, *Pharm. Eng. 12(2)*:48-51 (1992)).

Cloning of the meningococcal B class 3 protein gene (MB3)

The genomic DNA of Group B *Neisseria meningitidis* (strain 8765) served as the template for the amplification of class 3 porin (MB3) in a standard PCR. The amplified DNA fragment (930 b.p. long) of the mature porin protein was ligated in Nde I - BamH I cloning sites of the pET-24a cloning/expression

vector, originally constructed by Studier *et al.*, *J. Mol. Biol. 189*:113-130 (1986); *Meth. Enzymol. 185*:60-89(1990); *J. Mol. Biol. 219*:37-44 (1991), and manufactured by Novagen. The pET vectors were developed for cloning and for expressing target DNA fragments under the strong T7 transcription and translation signals. Expression from the T7 promoter is induced by providing the host cell with a source of T7 RNA polymerase. Newer, more convenient vectors utilizing the T7 expression system are now available from Novagen (Madison, WI 53711). The T7 expression system was successfully used for the expression of MB3 in *E. coli* (see Example 3).

The optimization of the translation elongation rate for the expressed MB3 gene

Codon usage is known to affect the translational elongation rate, and therefore it has been considered an important factor in affecting product yields (Romanos *et al.*, *Yeast 8*:423-488 (1992)). There is evidence that codon usage may affect both yield and quality of the expressed protein. A number of highly expressed genes show a strong bias toward a subset of codons (Bennetzen *et al.*, *J. Biol. Chem. 257*:3026-3031 (1982). This "major codon bias," which can vary greatly between organisms, is thought to be a growth optimization strategy. This mechanism allows an organism to be capable of efficient translation of highly expressed genes during rapid growth, as only a subset of tRNAs and aminoacyl-tRNA synthetases need to be present in high concentrations. Kurland *et al.*, *TIBS 12*:126-128 (1987). In cases where mRNA contains rare codons, aminoacyl-tRNAs may become limited, increasing the probability of amino acid misincorporations, and possibly causing ribosomes to drop off. Indeed, a high misincorporation frequency has recently been observed in a foreign protein produced in *E. coli* (Scorer *et al.*, *Nucleic Acids Res. 19*:3511-3516 (1991)). Moreover, proteins containing amino acid misincorporations are difficult to purify and may have both impaired activity and antigenicity. The presence of several rare codons has been shown to limit the production of tetanus toxin

fragment C in *E. coli* (Makoff *et al.*, *Nucleic Acids Res. 17*:10191-10201 (1989)). In yeast, Hoekema *et al.* (*Mol. Cell Biol. 7*: 2914-2924 (1987)) showed that substitution of a large proportion of preferred codons for rare codons in the 5′ portion of the PGK (phosphoglycerate kinase) gene caused a decrease in expression levels. Recently, the expression of an immunoglobulin kappa chain in yeast has been shown to be increased 50-fold when a synthetic codon-optimized gene is used, although the level of kappa chain mRNA remains the same.

Significant differences between codon usage profiles of *Pichia* and MB3 were found (Table 5). In order to optimize the translation efficiency, particularly at the beginning of translation elongation, codons optimal for *Pichia* were introduced into the 5′ region of the MB3 gene. When constructing the linker used to clone MB3 into pHIL-S1, the oligomers were synthesized so that they contained sequence optimized for *Pichia* expression. A 51 nucleotide long oligomer (51-mer) was synthesized for this purpose. The sequence of the oligomer is:

5'-TCGAGACGTCACTTTGTACGGTACTATTAAGGCTGGTGTTGAGA CTTCCCG-3'

A 47 nucleotide oligomer complementary to the 51-mer was also synthesized. The sequence of this oligomer is:

5'-CGGGAAGTCTCAACACCAGCCTTAATAGTACCGTACAAAGTGAC GTC-3'

These two oligomers, which contain *Xho*I and *Bsr*I restriction sites, were annealed to serve as a connector, and then ligated to vector pHIL-S1, which had been linearized with *Xho*I digestion. The ligated fragment was then digested with *BamH*I, gel purified, and ligated with an MB3 fragment obtained from cutting the pNV15 vector with both *Bsr*I and *BamH*I enzymes. The fragment was then cloned into the *Pichia* pHIL-S1 expression vector. The new DNA sequence of the 5′ region of MB3 was verified by DNA sequencing of pHIL-S1/MB3 isolated from *Pichia*.

The sequence of the original 5′ end of the gene for mature MB3 (from NT 1) is:

gac gtt acc ctg tac ggc acc att aaa gcc ggc gta gaa act tcc cgc tct gta ttt cac cag aac ggc
D V T L Y G T I K A G V E T S R S V F H Q N G

caa gtt act gaa gtt aca
Q V T E V T

The codon-optimized sequence of the same fragment (replaced nucleotides showed as capital letters), along with its corresponding amino acid sequence is:

gac gtC acT Ttg tac ggT acT att aaG gcT ggT gtT gaG act tcc cgc tct gta ttt cac cag aac
D V T L Y G T I K A G V E T S R S V F H Q N

ggc caa gtt act gaa gtt aca
G Q V T E V T

Vector pHIL-S1/MB3, containing the codon-optimized MB3 DNA, served as the template for the amplification of MB3 in a standard PCR. Oligomers were synthesized to serve as PCR primers. The PCR fragments of MB3 were inserted into *Pichia* expression vectors either directly or by using the Original TA Cloning Kit (Invitrogen); details are given below.

For the cloning of MB3 into the *EcoR*I site of pHIL-D2:

Forward primer (39 nt. having an engineered *EcoR*I site and a sequence (5'ATG) encoding an initiation methionine):

5'-CGAGAATTCATGGACGTCACTTTGTACGGTACTATTAAG-3'

Reverse primer (45 nt, having an engineered *EcoR*I site and stop codon):

5'-GCTGAATTCTTAGAATTTGTGGCGCAGACCGACACCGCCGGCAGT-3'

For the cloning of MB3 into the *EcoR*I-*Avr*II sites of pPIC9 and pPIC9: Forward primer (39 nucleotides (nt), having an engineered *EcoR*I site; no sequence encoding an initiation methionine was necessary because the leader peptide had an initiation methionine):

5'-AGCGAATTCGACGTCACTTTGTACGGTACTATTAAGGCT-3'

Reverse primer (36 nt, having an engineered *Avr*II site and stop codon):

5'-CACCCTAGGTTAGAATTTGTGACGCAGACCGACACC-3'

For PCR amplification of the complete MB3 gene, Vent® DNA polymerase (NEB) was used. The fidelity of this polymerase is 5-15-fold higher than that observed for Taq DNA polymerase. To generate an expression cassette plasmid, PCR fragments of MB3 (full length and truncated fragments) were inserted in *Pichia* expression vectors either directly or using the Original TA Cloning® Kit (Invitrogen), which includes a pCR™II vector for subcloning of PCR fragments. Direct cloning of DNA amplified by either Vent® DNA polymerase or *Pfu* DNA polymerase into the vector pCR™II is difficult, as the cloning efficiency is often very low. This is due to the 3' to 5' exonuclease proofreading activity of Vent® and *Pfu*, which removes the 3' A overhangs that are necessary for TA cloning, leaving blunt ends. The Original TA Cloning® Kit allows these blunt-ended fragments to be cloned. Use of this method eliminates any enzymatic modifications of the PCR product, and does not require the use of PCR primers containing restriction sites. To increase the cloning efficiency further, the Invitrogen protocol was modified as follows. Following amplification with Vent® or *Pfu* (*see* manual for The Original TA Cloning® Kit, protocol for the addition of 3'A-overhangs post amplification, p. 19), rather than placing the vial on ice, as recommended in the kit, the mineral oil in the PCR mixture was immediately removed using Parafilm™. This was accomplished by pouring the PCR mixture onto the Parafilm, and zigzagging the drop down the surface of the Parafilm with a gentle rocking motion until all of the oil had adhered to the Parafilm surface. The reaction mixture, now free of oil, was then collected into a fresh tube. The Invitrogen protocol was then resumed with the

addition of Taq polymerase. This method allowed the difficult cloning of PCR fragments into large expression vectors.

The expression cassette of the integrating vector (Invitrogen) contains the methanol-induced AOX1 promoter and its terminator, flanked by stretches of nucleotides up- and downstream from the AOX1 gene. The *P. pastoris* His4 gene served as an auxotrophic marker. These vectors do not contain a yeast *ori*, hence His^+ colonies must correspond to integration of the expression cassette. All PCR fragments of MB3 were inserted in frame with a *Pichia* Kozak consensus sequence (CAAAAAACAA) (Cavenor *et al. Nucleic Acids Res. 19*:3185-3192 (1991); Kozak *Nucleic Acids Res. 15*:8125-8148 (1987); Kozak *Proc. Natl. Acad. Sci. USA 87*:8301-8305 (1990)) to provide the best translation initiation of the MB3 gene. All inserts were placed under the control of the AOX1 promoter to drive expression of the gene of interest. After the ligation of the MB3 fragment in an appropriate expression vector, chemically competent *E. coli* cells were transformed (TOP 10F') (F'{*pro*AB, *lacI*^{q}, *lacZ*ΔM15, Tn*10* (Tet^R)} *mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC), ϕ80 *lacZ*ΔM15, Δ*lac*X74, *deo*R, *rec*A1, *ara*D139, Δ(*ara-leu*)7697, *gal*U, *gal*K, *rps*L(Str^R), *end*A1, *nup*Gλ$^-$). Other strains which may be suitable are DH5α F', JM109, or any other strain that carries a selectable F' episome and is *rec*A deficient (*endA* is preferable) (*Pichia* Expression Kit Instruction Manual, Invitrogen). Colonies with an MB3 insert were used for the preparation of CsCl purified maxi-prep of a plasmid DNA for *Pichia* transformation (Sambrook, J. *et al.*, Eds., *Molecular Cloning: A Laboratory Manual*. 2nd. Ed., Cold Spring Harbor Press (1989), pp. 1.42-1.43). Restriction analysis and DNA sequencing (DNA Sequencing Kit, Version 2 (USB)) confirmed that these constructs were correct.

Modification of the starting MB3 sequence was especially useful for intracellular expression of the porin gene (pHIL-D2/MB3 construct). Because the other constructs (pHIL-S1/MB3 and pPIC9/MB3) used for MB3 secretion contained codons optimal for *Pichia* in the leader peptide sequence upstream of the MB3 insert, the initiation of translation was not rate-limiting. In contrast, the

pHIL-D2 vector does not include any leader sequence and the initiation of translation must be started from the rare codons of the MB3 insert. The optimization of this sequence is believed to be responsible for the fact that pHIL-D2/MB3 constructs gave the highest level of MB3 expression of any of the clones tested (Tables 3, 4).

Transformation of yeast cells and DNA analysis of integrants

Plasmid DNA was linearized with single or double (for higher integration efficiencies) digestion, and *P. pastoris* strain GS115 ($his4^-$) was transformed to the His^+ phenotype by the spheroplast method using Zymolyase followed by adsorption of transforming DNA and penetration of this DNA through the spheroplast pores into the *Pichia* cells in the presence of PEG and Ca^{+2} (*Pichia* Expression Kit manual, Invitrogen, pp.33-38). By replica plating or patching on Minimal Dextrose (MD: 1.34% yeast nitrogen base (YNB - Difco), $4x10^{-5}$% biotin, 2% dextrose) versus Minimal Methanol (MM: 1.34% YNB, $4x10^{-5}$% biotin, 0.5% methanol), it was possible to determine which His^+ transformants also exhibited disruption of the *AOX1* gene. Transformed spheroplasts were seeded on agarose-containing plates using selective growth medium without histidine (MD). At the end of 4-6 days, white separated colonies of yeast transformants had appeared. These colonies were picked up and were seeded on selective methanol-containing medium (MM) for screening of AOX1-disrupted (Mut^S or Mut^-) transformants (*Pichia* Expression Kit manual, Invitrogen, p. 60).

Growth of the yeast and methanol induction

Because recombination events can occur in many different ways which affect the level of protein expression (clonal variation), at least 16 verified recombinant clones were screened to determine the level of MB3 expression. These colonies were grown in 5 ml of glycerol-containing Buffered Glycerol-

complex Medium (BMGY: 1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, $4x10^{-5}$% biotin, 1.0% glycerol) (*Pichia* Expression Kit manual, Invitrogen, p. 61) at 30°C in 50 ml 2098 Bluemax tubes (Falcon) in an Innova incubator shaker (New Brunswick Sci.) ("pilot" expression). After 1-2 days when cultures had reached an OD600 = 5-10, the cells were harvested by centrifugation (4000 rpm for 10 minutes at room temperature) and were resuspended in methanol-containing Buffered Methanol-complex Medium (BMMY: 1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, $4x10^{-5}$% biotin, 0.5% methanol) (*Pichia* Expression Kit manual, Invitrogen, p. 61) for the induction of the *AOX1* promoter. To replenish exhausted methanol, 0.5% of fresh methanol was added each day to induced cells. Aliquots of the cells were collected every day for 6 days by centrifugation, and stored (pellets and supernatants separately) at -70°C before examining. The most promising clones were examined for the optimization of protein expression and to scale-up the expression protocol to produce more protein.

Lysis of* P. pastoris *cells, analysis by SDS-PAGE and Western blot analysis

Cells were broken by agitation in breaking buffer (50 mM sodium phosphate, pH 7.4; 1 mM PMSF(phenylmethylsulfonyl fluoride), 1 mM EDTA and 5% glycerol). Equal volumes of acid-washed glass beads (0.5 mm in diameter) were added. The mixture was vortexed for a total of 4 min, 30 sec mixing each, followed by 30 sec on ice. The soluble fraction was recovered by centrifugation for 10 min at 14000 rpm at 4°C. Supernatant (or cell lysate, or fraction of "soluble" proteins) was removed and stored at -70°C, and the residual cell pellet was extracted by vortexing with SDS sample buffer (1% SDS, 5% beta-mercaptoethanol, 10% glycerol, 10 mM EDTA, 0.025% bromophenol blue) followed by boiling for 10 min. Lysates were centrifuged again and the aqueous layer was examined as fraction of "insoluble" or membrane associated proteins.

NOVEX pre-cast 8-16% gradient gels were used for separation of proteins according to the procedure of Laemmli *(Nature 227*:680-685 (1970)). Proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were stained with Coomassie Brilliant Blue R250, or were transferred to polyvinylidene difluoride (PVDF) membrane using a Transblott apparatus (BioRad Laboratories) according to the company specification.

The Western blot procedure was carried out without detergents, using only blocking procedures, as described by Sheng and Schuster (*Bio Technique 13*:704-708 (1992)) with some modifications. This method provides high specificity and sensitivity with a low background. For the transfer, both Western transfer membrane and the SDS-PAGE separating gel were equilibrated with transfer buffer (24mM Tris-HCl/192 mM glycine/ 20% methanol) for 20 minutes prior to electrotransfer. The transfer was performed at 90V and 4°C for 3-4 hours. Transfer of proteins to PVDF membranes was monitored by the transfer of prestained molecular weight markers (BRL).

Immunostaining of proteins was carried out as follows. The transfer membrane was rinsed with TBS (10mM Tris-HCl/.09% NaCl, pH 7.2). The membrane was then incubated in 1% non fat dried milk PBS solution (M-PBS) with .02% sodium azide at 37°C for 3 hours (or at 4°C overnight). The membrane was then washed 3 times with TBS/0.5% BSA (BSA/TBS) and once with TBS. The membrane was then incubated with the primary mouse anti-MB3 antibody (mouse polyclonal antisera against purified OMP class 3) diluted to about 1:4000 in PBS/1%BSA (BSA/PBS), and the membrane was again washed 3 times with TBS/0.5% BSA (BSA/TBS) and once with TBS. The membrane was then incubated in 1% M-PBS at room temperature for 30 minutes with gentle shaking. The membrane was washed 3 times with TBS/0.5% BSA (BSA/TBS) and once with TBS. The membrane was then incubated in the secondary alkaline phosphatase-conjugated anti-mouse antibody (Kirkegaard & Perry Laboratory (KPL), Gaithersburg, MD) diluted 1:4000 in 1% BSA/PBS. The membrane was then washed 2 times with 0.5% BSA/TBS and 3 times with .25% Tween 20 in

PBS. These washing steps differed from those recommended by Sheng and Schuster; the improved protocol provided less background than did the wash steps of the reference, which utilized 6 washes in 0.5% BSA/PBS. The membrane was then incubated in alkaline phosphatase buffer (0.05% M Tris-HCl, pH 9.5; 10 mM $MgCl_2$), followed by incubation in BCIP/NBT substrate solution (KPL). The development was stopped by washing the membrane in PBS/50 mM EDTA. The limit of detection was about 2-5 ng of native MB3 protein.

Results and discussion

The strategy used to insert the cDNA encoding the mature MB3 into expression vectors and the steps using this construct for the transformation of *P. pastoris* are outlined below. First, the MB3 gene is cloned into one of the 4 *Pichia* expression vectors. In the next step, the resulting construct is linearized by digestion with *Not*I or *Bgl*II, and *his4 Pichia* spheroplasts are transformed with the linearized construct. In the following step, a recombination event occurs *in vivo* between the 5´ and 3´*AOX1* sequences in the vector and in the genome, resulting in replacement of the *AOX1* gene with the MB3 gene. Next, the *Pichia* transformants are selected on histidine-deficient medium, on which only cells that have undergone gene replacement can grow. The one-step gene replacement method described for *S. cerevisiae* (Rothstein, *Meth. Enzymol. 101*:202-211 (1983)) was successfully used by Cregg *et al.* (*Biological Research on Industrial Yeast, Vol. II*, Stewart *et al., eds.*, CRC Press, Boca Raton, pp.1-18 (1987)) for the replacement of the *P. pastoris* AOX1 structural gene. Transformation of GS115 with 10 μg of linearized expression vectors (pHIL-D2, pHIL-S1, pPIC9, and pPIC9K) with MB3 insert gave more than 100 colonies in each experiment. Thus, the procedure yielded $>10^2$ His$^+$ colonies per μg DNA, which is comparable to that reported for the best results of *P. pastoris* transformations. These transformants have the ability to grow on histidine-deficient medium (MD-minimal dextrose), and so are His$^+$. About 10-40% of

these recombinants were "methanol slow" (Mut^s -- "methanol utilization slow"), i.e., demonstrated impaired growth on media such as MM (minimal methanol), which contains methanol as the sole carbon and energy source. These His$^+$/Mut^s transformants are a result of the replacement of the AOX1 structural gene with the MB3 expression cassette containing the His$^+$ gene via a double crossover event. Recombination events may also occur as integration or insertion (single crossover events) of the expression cassette into the 5' or 3' AOX1 region, which leaves the AOX1 gene intact. Among the His$^+$/Mut^s clones, 25-35% were positive, MB3-expressing transformants (Table 2). The reason that the *AOX1*-deleted transformants grow at all on methanol medium is due to low-level expression of alcohol oxidase activity by the *AOX2* gene product. Analysis of DNA isolated from these "positive" recombinants using PCR with 5' *AOX1*, 3' *AOX1*, 5' MB3, 3' MB3 and other specific primers, indicated that the *AOX1* structural gene was indeed replaced by the fragment containing the MB3 and *HIS4* genes. Analysis of the DNA isolated from His$^-$/Mut$^+$ transformants indicated that the *AOX1* structural gene was intact and that the entire vector containing His4 DNA had integrated elsewhere. Among 39 *AOX1*-disrupted transformants that expressed MB3, no His$^+$/Mut$^+$ transformants were found, indicating preference for the *AOX1* replacement mode of integration.

The results of immunoblot analysis of 84 *Pichia* transformants indicated that one may express the MB3 protein using all of the constructed recombinant plasmids, pHIL-D2/MB3, pHIL-S1/MB3, pPIC9/MB3, and pPIC9K/MB3 (Table 3). Thirty-nine clones were isolated that expressed the MB3 protein. Antigenic specificity of expressed MB3 protein was examined and was confirmed by Western blot analysis using monoclonal and polyclonal antibodies raised against wild type *N. meningitidis* OMP class 3. These results led to the conclusion that all of the expression vectors were correctly constructed, and that the transformations of *Pichia* spheroplasts were properly performed.

The amount of expressed MB3 was determined by densitometric scanning of the Coomassie brilliant blue stained protein bands fractionated by SDS-PAGE

using a Model GDS-7500 scanning densitometer (UVP Life Sci.) or Model IS-1000 densitometer (Alpha Innotech Corp.). Purified OMP class 3 extracted wild type of *N. meningitidis* was used as a standard. Based on the results (summarized in Table 3), the level of protein expression was estimated to be moderate to high.

The optimization of the translation elongation rate for the expression of the MB3 gene (see Materials and Methods, above) was very useful. The modification of the starting MB3 sequence was especially effective for intracellular expression of the porin gene (pHIL-D2/MB3 construct). Because other constructs (pHIL-S1/MB3 and pPIC9/MB3, both used for MB3 secretion) contained codons optimal for *Pichia* in the leader peptide sequence upstream of the MB3 insert, the initiation of translation of these cassettes was not rate-limiting. In contrast, the pHIL-D2/MB3 construct did not include a leader sequence, and so without codon optimization, translation would have had to have been initiated at rare codons of the MB3 insert. The codon-optimized pHIL-D2/MB3 construct, when transformed into *Pichia* chromosomal DNA, provided the highest level of MB3 expression of all the other mentioned MB3 expression constructs (Tables 3 and 4). Thus, this modification of the translation start sequence of MB3 appears to be responsible for the high yield of expressed protein in pHIL-D2/MB3 constructs.

The level of MB3 expression by the best clones (*Pichia* transformed with the pHIL-D2/MB3 construct) was in the range of 0.1-0.6 g per 1L of cell suspension, or 1-3 mg per g of cell pellet (Table 3, Fig. 12). Such efficiency of expression in yeast has been reported for many of the following manufactured proteins: hepatitis B surface antigen (0.3 g/L), superoxide dismutase (0.75 g/L), bovine and human lysozyme (0.3 and 0.7 g/L, respectively), human and mouse epidermal growth factors (0.5 and 0.45 g/L respectively), human insulin-like growth factor (0.5 g/L), human interleukin-2 (1.0 g/L), aprotinin analog (0.8 g/L), Kunitz protease inhibitor (1.0 g/L), etc. (Cregg *et al.*, *Biotechnology*, *11*:903-906, Table 1 (1993)).

It should be emphasized that all of the previously listed levels of expression for manufactured proteins are the result of production of these proteins during fermentation in high cell density fermentors. MB3 was expressed utilizing only shake flask cultures which, as a rule, provide much lower expression levels than does fermentation. Recently reported observations lead one to expect a much higher yield (a 5-10 fold or greater increase) of MB3 in a fermenter (Cregg *et al.*, 1993). *P. pastoris* adapts well to being scaled up from shake flask to high density fermentor cultures. In addition, where *AOX*-deleted *Pichia* strains are used for fermentation, production of foreign proteins can be optimized by first causing rapid growth, and then adding methanol to induce protein production while minimizing additional cell growth. The long amount of time needed to produce proteins when *Pichia* is growing on methanol can be reduced by applying one of several mixed-feed fermentation strategies (Siegel *et al.*, *Biotechnol. Bioeng. 34*:403-404 (1989); Brierley *et al.*, Int. Patent Application No. WO 90/03431 (1989); Brierly *et al.*, *Biochem. Eng. 589*:350-362 (1990); Siegel *et al.*, Int. Patent Application No. WO 90/10697 (1990)).

Another promising aspect of the expression levels of MB3 protein in *Pichia* is that the results were similar for all examined clones. As other investigators have found that in shake flask induction the level of expression is proportional to the number of copies of inserted gene of interest (Clare *et al.*, 1991), it can be deduced that all of the MB3 clones tested were single-copy chromosomal integrants, and thus that no *Pichia* recombinants with multiple integrated copies of the MB3 fragment were isolated.

An important factor in obtaining high levels of expression using *P. pastoris* is the ability to obtain recombinants with multicopy transplacement or integration (Romanos *et al.*, *Vaccine 9*:901-906 (1991); Clare *et al.*, *Bio/Technology 9*:455-460 (1991); Clare *et al.*, *Gene 105*:205-121 (1991)). . Multicopy transformants have been found to be surprisingly stable over multiple generations during growth and induction in high cell density fermentations. Since this multiple gene insertion event occurs at a low frequency during

spheroplast transformation, a special dot blot screening of a number of recombinants is used (Scorrer *et al.*, *Bio/Technology 12*:181-184 (1993)). An alternative to screening for spontaneous multiple insertion events is to insert multiple copies of the gene(s) of interest into *Pichia* expression vector pAO815, which has recently been constructed by Invitrogen for this purpose.

Before attempting to express MB3, the protein was evaluated to determine if any of the factors believed to reduce expression levels were present. One of the factors which can reduce expected high-level accumulation of a protein is proteolytic stability. It is now known that highly expressed proteins are devoid of good PEST sequences. PEST sequences contain proline (P), glutamic acid (E), serine (S) and threonine (T), and are found in all rapidly degraded eukaryotic proteins of known sequence; such proteins have been implicated as favored substrates for calcium-activated proteases (Rogers *et al.*, *Science 234*:364-369 (1986)). Proteins that are expressed at high levels in yeast do not contain a so-called "good" PEST sequence (having a score >5 as calculated by the algorithm developed by Rogers *et al.* (1986)), which leads to susceptibility to proteolysis, nor do they contain the pentapeptide sequences XFXRQ or QRXFX (X=any amino acid), which are selective for degradation of cytoplasmic proteins by the lysosomal pathway (Dice, J.F., *Fed. Am.Soc. Exp. Biol. (FASEB) J. 1*:349-357 (1987)). Proteins that are expressed at high levels in yeast do not contain these pentapeptide sequences. Computer analysis of the MB3 sequence identified a "poor" but not "good" PEST region (13-32aa) having the sequence (ETSRSVFHQNGQVTEVTTAT. According Rogers *et al.* (1986) such a poor PEST sequence weakly influences the proteolytic stability of eukaryotic proteins. Thus, one of the factors which leads to proteolysis is not present in MB3.

MB3 also does not contain the highly conserved pentapeptide sequences mentioned above. The sequence RQSFI (75-79aa) is present in MB3: this sequence displays some homology to the degradation pentapeptide QRXFX, but is not believed to greatly destabilize MB3.

The nature of the NH_2-terminal amino acid residue can also be an important factor in the susceptibility of a protein to degradation. Varshavsky *et al.* have demonstrated that the presence of certain amino acids at the NH_2-terminus provide a stabilizing effect against rapid degradation by ubiquitin-mediated pathways (the N-end rule pathway) (Varshavsky *et al. Yeast Genetic Engineering*, Butterworths, pp. 109-143 (1989)). Most proteins that are expressed at high levels in yeast have a stabilizing amino-terminus amino acid residue (A, C, G, M, S, T or V). Examples of such proteins include human superoxide dismutase, human tumor necrosis factor, phosphoglycerate kinase from *S. cerevisiae*, invertase from *S. cerevisiae*, alcohol oxidase from *P. pastoris*, and extracellular alkaline protease from *Y. lipolytica* (Sreekrishna *et al.*, *Biochemistry 28*:4117-4125 (1989)). Although MB3 is expressed well in yeast, the NH_2-terminal aspartic acid (D) of MB3 does not provide a stabilizing effect against rapid degradation by ubiquitin-mediated pathways.

It is possible that the NH_2-terminal aspartic acid of MB3 will play a role in the level of MB3 produced from *Pichia* in large scale production. Replacing the first amino acid of MB3 with one of the amino acids known to stabilize the NH_2-terminus of proteins, mentioned above, could improve the level of MB3 production.

It was decided to proceed with experiments attempting to express MB3 in yeast, as most of the factors known to reduce expression levels were not present in MB3.

The best expression of MB3 was provided by *Pichia* clones transformed with the pHIL-D2/MB3 expression cassette (Tables 3 and 4). This pHIL-D2 vector generated intracellular expression of complete, monomeric, non-fusion, non-secreted MB3 with an expected MW of about 34 kDa. These clones provided the highest level of expression of MB3, up to 600 mg/L or 3 mg per g of wet cell pellet (Table 4). About 90-95% of this product was insoluble, membrane-associated material, *i.e.*, material which sediments upon centrifugation for 5 min at 10,000g, and that can be extracted by treatment with SDS-

containing buffer (PAGE sample buffer) followed by boiling. The protein can then be renatured to a conformation that can be easily recognized by an anti-meningococcal OMP class 3 antibody.

Induction of pHIL-D2/MB3 constructed clones with methanol resulted in the rapid expression and fast accumulation of intracellular MB3. After 24 hours of a methanol induction, the level of expressed MB3 was estimated at not less than 80% of maximal, which was reached after 5-6 days.

The pHIL-D2/MB3-containing *Pichia* recombinant is the most promising for commercial production. This clone provides relatively high levels of expression which could be significantly improved by using multiple-copy recombinants, and by producing the protein in a fermentor. The fact that MB3 is rapidly produced also provides an advantage for large scale manufacture.

MB3 expressed in an intracellular form was purified by a denaturation/renaturation protocol, followed by gel filtration and ion exchange chromatography. The resultant purified protein exhibits an elution profile on size exclusion chromatography that resembles the recombinant class 3 protein overexpressed in *E. coli*. MB3 expressed by either *E. coli* or *P. pastoris* co-elutes with the native wild-type counterpart, indicating that MB3 expressed by either *E. coli* or *P. pastoris* refolds and oligomerizes, achieving full native conformation (Figs. 14A and 14B).

Both the native (*Pichia*) secretion signal (PHO1) and the alpha-factor signal sequence from *S. cerevisiae* were tested for targeting expressed porin to the secretary pathway. Unexpectedly, the shorter PHO1 leader was more effective for causing MB3 secretion. The pHIL-S1 *Pichia* transfer vector includes a sequence encoding the 2.5 kDa PHO1 leader peptide, a secretion signal peptide of *P. pastoris*. In the pHIL-S1/MB3 construct, the sequence encoding MB3 was inserted downstream of the PHO1 leader sequence. 40-50% of the 36.5 kDa expressed fusion protein PHO1/MB3 produced by pHIL-S1/MB3 clones was properly cleaved to generate a 34 kDa MB3 monomer (Tables 2 and 3), and 5-10% of expressed soluble porin was secreted. The pPIC9 and pPIC9K

Pichia transfer vectors include a sequence encoding the 10 kDa alpha-factor leader derived from *S. cerevisiae*. *Pichia* clones transformed by pPIC9/MB3 or pPIC9K/MB3 did not secrete porin. These recombinants expressed a 44 kDa alpha-factor prepro/MB3 fusion protein well, but no evidence of correct cleavage and processing was observed. Improved secretion of expressed MB3 was not obtained by using its 3' truncated fragment fused with either PHO1 leader or alpha-factor leader peptides.

Example 10. Isolation, purification and characterization of MB3 protein expressed as a secretory protein

Yeast cells cultures harboring the expression vector containing the gene for MB3 (pHIL-S1-pNV318) were configured to isolate the protein as soluble secreted material). The supernatant was clarified by precipitation with 20% ethanol (v/v) to remove contaminating yeast culture impurities. The supernatant was then precipitated with 80% ethanol (v/v). The resulting pellet was washed with TEN buffer (Tris HCl, pH 8.0, 100 mM NaCl and 1 mM EDTA), in order to remove other hydrosoluble contaminating secreted proteins. The pellet containing MB3 was dissolved in an aqueous solution of detergent (solubilizing buffer), comprised of TEN buffer with 5% Z 3-14. The solution was applied to a Hi-Trap Q Sepharose ion exchange column (1 ml) (Pharmacia) equilibrated in 50 mM Tris, 0.2 M NaCl and 1.0 mM EDTA (pH 8.0). A gradient of 0.2-1.0 M NaCl was applied, and MB3 protein eluted as a single peak.

Example 11. Isolation, purification and characterization of MB3 protein expressed as an insoluble-membrane bound protein

Yeast cells cultures harboring the expression vector containing the gene for MB3 (pHILD-2--pNV322) (see Table 3) were resuspended in breaking buffer

(i.e., 50 mM sodium phosphate buffer, pH 7.4, 1 mM EDTA, and 5% glycerol), to a concentration equivalent to 50-100 ODs. The suspension was added to the same volume of acid treated glass beads. The suspension was lysed using a Minibead-Beater (Biospec Products, Bartlesville, OK), in 8 consecutive cycles of 1 min each, followed by 1 min on ice, between each cycle. As an alternative procedure, the lysis process was facilitated by the addition of Zymolase to the breaking buffer. The suspension was transferred to a glass sintered filter to separate the glass beads, and the cell suspension was collected in the filtrate. The beads were further washed and the filtrates combined. The suspension was then centrifuged at 12,000 rpm for 15 min at 4°C. A series of consecutive washing steps was applied to the resultant pellet, consisting of the following: (a) TEN (Tris HCl, pH 8.0, 100 mM NaCl, and 1 mM EDTA) containing 0.5% deoxycholate; (b) TEN containing 0.1% SDS and 1% Nonidet, after which the suspension was rotated for 30 min at 25°C; (c) washing with TEN buffer; and (d) washing with TEN buffer containing 5% Z 3-14, under rotation overnight at 4°C. Each washing step was followed by centrifugation at 12,000 rpm for 10 min at 4°C to collect the pellet for the following step. As an alternative method of washing the pellet, the suspension was passed through an 18 gauge needle in lieu of rotation in steps (b) and (d). Finally, the MB3 was extracted with 8M urea, or 6M guanadinium HCl, and the extract was sonicated for 10 min, using a water bath sonicator. The extract was clarified by centrifugation (12,000 rpm, for 10 min at 4°C), the same volume of a 10% aqueous solution of 3,14-zwittergen (Calbiochem) was added and the solution thoroughly mixed. The solution was again sonicated for 10 min. Any residual material was removed by centrifugation. This mixture was then applied to a Sephacryl S-300 (5x100 cm) column (Pharmacia) equilibrated in a buffer comprised of 0.1 M Tris-HCl, 0.2 M NaCl, 10 mM EDTA, 20 mM $CaCl_2$ and 0.05% Z 3-14 (pH 8.0). Fractions containing class 2 protein were identified by SDS-PAGE, pooled, and applied to a Hi-Trap Q Sepharose ion exchange column (1 ml) (Pharmacia) equilibrated in 50 mM Tris, 0.2 M NaCl and 1.0 mM EDTA (pH 8.0). A gradient of 0.2-1.0 M

NaCl was applied and MB3 protein eluted as a single peak. Figures 14A, 14B and 15 depict the elution profile of purified MB3 in a Sepharose 12 (Pharmacia) connected to an HPLC (Hewlett Packard, model 1090). Based on the comparison with the native wild-type class 3 protein, as well as calibration using molecular weight standards, the elution profile is indicative of trimeric assembly.

Example 12. Preparation of GAMP-TT Conjugate

12.1 Preparation of NMA polysaccharide for conjugation. *N. meningitidis* group A (NMA) strain 604 A was grown in modified Franz medium (Franz, I. D., *J. Bact. 73*:757-761 (1942). Precipitation of the polysaccharide as a cationic detergent complex followed by fractional precipitation with ethanol provided the high molecular NMA capsular polysaccharide. The high molecular weight polysaccharide was further purified by ultra filtration. Partial hydrolysis of the polysaccharide with 100 mM sodium acetate buffer pH 5.0 at 70°C yielded a low molecular weight polysaccharide in the range of 10,000-20,000 daltons. The free reducing terminal residue of the polysaccharide was reduced with $NaBH_4$ in the cold to preserve O-acetyl substituents and then oxidized with sodium periodate to generate terminal aldehyde groups. The oxidized polysaccharide was the purified and fractionated by size exclusion chromatography to provide activated group A meningococcal polysaccharide (GAMP) of average molecular weight about 13,000 daltons.

12.2 Preparation of GAMP-TT conjugate. Tetanus toxoid (Serum Statens Institute, Denmark) was first purified to its monomeric form (mw 150,000) by size exclusion chromatography using a Superdex G-200 column (Pharmacia). Freeze-dried tetanus toxoid monomer (1 part by weight) and oxidized GAMP (2.5 part by weight) were dissolved in 0.2 M phosphate buffer pH 7.5. Recrystallized $NaBH_3CN$ (1 part) was added and the reaction mixture incubated at 37°C for 4 days. The conjugate was purified from the free components by size exclusion chromatography using a Superdex G-200 column

(Pharmacia), and PBS containing 0.01% thimerosal as an eluent. Purified GAMP-tetanus toxoid conjugate was stored at 4°C in this buffer. The polysaccharide content of the conjugate based on phosphorus analysis (Chen assay) was about 18-20% by weight.

Example 13. *Preparation of GCMP-TT Conjugate*

13.1 ***Preparation of NMC polysaccharide for conjugation.*** The capsular polysaccharide was isolated from the growth medium of *Neisseria meningitidis* group C (NMC) strain C 11. This strain was grown in modified Franz medium. The NMC polysaccharide (group C meningococcal polysaccharide (GCMP)) was isolated from the culture medium by cetavlon precipitation as described for the GAMP. Native GCMP was O-deacetylated with base and depolymerized by oxidative cleavage with $NaIO_4$ to an average molecular weight of 10,000-20,000. The cleaved polysaccharide was sized and purified by gel filtration chromatography to provide a highly purified product of average molecular weight about 12,000 daltons and having aldehyde groups at both termini.

13.2 ***Preparation of GCMP-TT conjugate.*** Tetanus toxoid monomer (1 part) and solid oxidized GCMP (1 part) were dissolved in 0.2 M phosphate buffer pH 7.5 and incubated at 37°C with 1 part of recrystallized $NaBH_3CN$ for 4 days. The conjugate was purified from its free components by gel filtration chromatography on Superdex G-200 using PBS containing 0.01% thimerosal as eluent. The purified conjugate was stored at 4°C prior to being formulated for animal studies. The content of the polysaccharide in the conjugate was 33% based on its sialic acid content as measured by the Svennerholm resorcinol assay (*Biochim. Biophys. Acta 244:*604-611 (1957).

Example 14. *Preparation of N-Propionyl Group B Meningococcal Polysaccharide-rPorB Conjugate*

14.1 Preparation of Neisseria rPorB. Expression of class 3 *N. meningitidis* porin protein (PorB) in *E. coli* and purification of porin gene products is described *supra*. The recombinant rPorB protein was purified by using a sephacryl S-300 molecular sieve column equilibrated with 100 mM Tris-HCl, 200 nM NaCl, 10 mM EDTA, 0.05% Zwittergen 3, 14 (Calbiochem. La Jolla, CA), 0.02% sodium azide pH 8.0. The protein fractions as measured by their OD_{280} eluting with an apparent molecular weight of trimers were pooled and diafiltered against 0.25 M HEPES, 0.25 M NaCl, 0.05% Zwittergen 3, 14 pH 8.5, to a concentration of 10-12 mg/ml.

14.2 Preparation of N-propionylated Group B Meningococcal Polysaccharide (GBMP). The N-propionylated GBMP and its oxidized form were prepared as described in U.S. Patent No. 4,727,136 and EPO 0504202 , both of which are fully incorporated by reference herein.

14.3 Preparation of N-Pr-GBMP-rPorB conjugate. To 10 mg of oxidized N-Pr-GBMP of average molecular weight 12,000 was added 33 µl of a 12 mg/ml of rPorB protein in 0.25 M HEPES, 0.25% M NaCl, 0.05% Zwittergen 3, 14, pH 8.5. The solution was mixed until all solid dissolved and 6.5 mg of recrystallized $NaBH_3CN$ was added. The solution was incubated at 37°C for 4 days and the conjugate was purified from the mixture by using a Superdex G-200 column (Pharmacia) equilibrated with PBS -0.0% thimerosal. Protein fractions were combined and stored at 4°C. The conjugates were analyzed for their sialic acid content by the resorcinol assay and for protein with the Pierce Coomassie Plus assay. The resulting conjugate had a polysaccharide content of about 20-25% and is devoid of any pyrogens as measured by the LAL and rabbit pyrogenicity tests.

Example 15. Analysis of Conjugates by Capillary Electrophoresis

15.1 System and method. Analysis was performed by Capillary Zone Electrophoresis on a Beckman 2000 Series CE system (Beckman Instruments Inc., Fullerton, CA) using an untreated fused silica capillary of dimensions 47 cm total length (40 cm effective length) by 50 μm i.d. (375 μm o.d.) and 0.4N borate buffer, pH 10.2 as electrolyte (Hewlett Packard, Palo Alto, CA). System control and data acquisition was performed using Beckman Gold system software. The voltage was set at 25 KV and the detector was set to 200 nm detection wavelength. The capillary temperature was set to 20°C. The capillary was conditioned between runs with a high pressure rinse for 2.0 minutes with 0.1M sodium hydroxide followed by 2.0 minutes with deionized water. All samples were pressure injected. All buffer and sample media were filtered through an appropriate 0.2 μm membrane filter and degassed prior to use.

15.2 Analysis of Conjugates. After purification the conjugates were concentrated by ultrafiltration through an Amicon Centricon-3 concentrator (Amicon, Inc., Beverly, MA). Meningococcal polysaccharide and tetanus toxoid monomer calibration samples were prepared in deionized water at a concentration of 0.25 mg/ml and 0.28 mg/ml, respectively. The method was determined to be selective for the glycoprotein and conjugate components with adjacent components being completely separated (Rs>1.5), as demonstrated in the electropherograms of the polysaccharides and protein spiked glycoprotein conjugates (Fig. 20 and Fig. 21). Fig. 20 shows the GAMP-TT conjugate spiked with GAMP and TT-monomer conjugate components, while Fig. 21 shows the GCMP-TT conjugate spiked with GCMP and TT-monomer conjugate components. The lower limit of detection (LLD) for the free form polysaccharide and protein components for the method was determined to be in the subnanogram level. A lower limit of quantitation (LLQ) of approximately 0.6 ng was obtained for the free form of each component. A linear response was obtained for the selected total mass of each component. A linear response was obtained for the

selected total mass range of 0.6-2.6 ng and 0.6-2.4 ng for the polysaccharide and protein, respectively, with a coefficient of determination of 0.99 for both curves. Using this CZE based assay, analysis of a meningococcal polysaccharide-tetanus toxoid conjugate indicated a free polysaccharide content of less than about 5% and a free protein content of less than about 2%.

Example 16. Immunization and Immunoassays

16.1 Trivalent conjugate vaccine formulation. Each individual conjugate component (A, B, C) was absorbed onto Aluminum hydroxide ($Al(OH)_3$) Alhydrogel (Superfos, Denmark) at a final Al concentration of 1 mg/ml of the trivalent vaccine. Three vaccines were formulated in which the doses of each conjugated polysaccharide varied. Formulations had either about 2 μg of each A, B, and C conjugated polysaccharide; or about 2 μg A conjugated polysaccharide, about 5 μg B conjugated polysaccharide and about 2μg C conjugated polysaccharide; or about 5 μg of each A, B, and C conjugated polysaccharide per dose of 0.2 ml of PBS, 0.01% thimerosal.

16.2 Immunization. Female Balb/c mice (Charles River Laboratories) 4-6 weeks old, were injected i.p. at days 0, 28, and 42. Bleeds were performed at days 0, 14, 28, and 42, and mice were finally exsanguinated at day 52. Sera were stored at -70°C prior to serological analysis.

16.3 Immunoassays:

ELISAs: Antibody titers to each A, N-propionylated B and C polysaccharides were determined by ELISA using the corresponding HSA conjugates as coating antigen (Figs. 22, 23, and 24). Antibody titer was defined as the x-axis intercept of the linear regression curve of absorbance vs. absorbance x dilution factor.

Bactericidal Assays: Bactericidal assays were performed using baby rabbit serum as a source of complement and *N. meningitidis* strains H 44/76 (Serotype 15), C11 and A1 respectively used as group B meningococcal, group C meningococcal, and group A meningococcal organisms in this assay (Figs. 25,

26, and 27). Bactericidal titer was defined as the serum dilution producing 50% reduction in viable counts.

Having now fully described this invention, it will be understood by those of ordinary skill in the art that the invention can be practiced within a wide and equivalent range of conditions, formulations and other parameters without affecting the scope of the invention or any embodiment thereof. All patents and publications cited herein are fully incorporated by reference herein in their entirety.

Table 1. ELISA and Bactericidal Titers of Group B Meningococcal Conjugate Vaccines (N-Pr GBMP-Protein)

Vaccine	Adjuvant	ELISA Titer	Bactericidal Titer
N-Pr GBMP-TT	Saline	5,400	0
	$Al(OH)_3$	13,000	0
	ST[1]	17,000	0
	CFA[2]	40,000	800
N-Pr GBMP-PP	Saline	20,000	500
	Saline	22,000	150
	Saline	39,000	960
	$Al(OH)_3$	93,000	200
	$Al(OH)_3$	166,000	>3,200
	$Al(OH)_3$	130,000	1,200
	ST	53,000	1,000
	ST	29,000	1,700
	ST	72,000	1,500
N-Pr GBMP	Saline	>100	0
	$Al(OH)_3$	>100	0
	ST	>100	0
PP	Saline	>100	0
	$Al(OH)_3$	>100	0
	ST	660	0

[1]ST = Stearyl tyrosine.

[2]CFA = Complete Freund's Adjuvant

Table 2. Efficacy of a transformation of yeast (Pichia) cells

Construct	Number of analyzed transformants	MB3 expressed transformants	
		Number of positive	% from total
pHIL-D2 / MB3	32	9	28
pHIL-S1 / MB3	23	8	35
pPIC9 / MB3	16	4	25
pPIC9K / MB3	16	5	31

Table 3. Expression of MB3 porin protein with recombinant *Pichia pastoris*

Code AMVAX	Clone	Vector	Level of expression mg / g	mg / L	Secretion
pnv 311	S1/MB3/3/s	pHIL-S1	ND	20 - 30	0
pnv 312	S1/MB3/5/s	pHIL-S1	ND	30 - 40	0
pnv 313	S1/MB3/7/s	pHIL-S1	ND	30 - 40	0
pnv 314	S1/MB3/12/s	pHIL-S1	ND	20 - 30	5 - 10
pnv 315	S1/MB3/15/s	pHIL-S1	ND	20 - 30	0
pnv 316	S1/MB3/18/s	pHIL-S1	ND	80 - 100	5 - 10
pnv 317	S1/MB3/22/s	pHIL-S1	ND	50 - 60	5 - 10
pnv 318	S1/MB3/23/s	pHIL-S1	ND	300 - 400	5 - 10
pnv 321	D2/MB3/1-7/s	pHIL-D2	2.4	480	0
pnv 322	D2/MB3/2-1/s	pHIL-D2	3.0	600	0
pnv 323	D2/MB3/2-6/s	pHIL-D2	1.7	340	0
pnv 324	D2/MB3/2-8/s	pHIL-D2	1.6	320	0
pnv 325	D2/MB3/4-1/s	pHIL-D2	1.7	340	0
pnv 326	D2/MB3/4-3/s	pHIL-D2	2.4	480	0
pnv 327	D2/MB3/4-4/s	pHIL-D2	2.4	480	0
pnv 328	D2/MB3/4-5/s	pHIL-D2	2.4	480	0
pnv 329	D2/MB3/4-26/s	pHIL-D2	2.4	480	0
pnv 341	P9/MB3/1-46/s	pPIC-9	ND	10 - 20	0
pnv 342	P9/MB3/1-261/s	pPIC-9	ND	80 - 100	0
pnv 343	P9/MB3/1-263/s	pPIC-9	ND	20 - 30	0
pnv 344	P9/MB3/1-268/s	pPIC-9	ND	20 - 30	0
pnv 345	9K/MB3/Tr/3-4/s	pPIC-9K	ND	150 - 200	5
pnv 346	9K/MB3/Tr/3-5/s	pPIC-9K	ND	100 - 150	0
pnv 347	9K/MB3/Tr/3-6/s	pPIC-9K	ND	100 - 150	0
pnv 348	9K/MB3/Tr/3-8/s	pPIC-9K	ND	80 - 100	0
pnv 349	9K/MB3/Tr/3-9/s	pPIC-9K	ND	80 - 100	0

Code AMVAX	Clone	Vector	Level of expression mg / g	mg / L	Secretion
pnv 350	9K/MB3/6-1/s	pPIC-9K	ND	150 - 200	0
pnv 351	9K/MB3/6-2/s	pPIC-9K	ND	100 - 150	0
pnv 352	9K/MB3/6-3/s	pPIC-9K	ND	100 - 150	0
pnv 353	9K/MB3/6-5/s	pPIC-9K	ND	80 - 100	0
pnv 354	9K/MB3/6-9/s	pPIC-9K	ND	80 - 100	0
pnv 355	9K/MB3/8-22/s	pPIC-9K	ND	150 - 200	0
pnv 356	9K/MB3/9-5/s	pPIC-9K	ND	80 - 100	0
pnv 357	9K/MB3/10-20/s	pPIC-9K	ND	80 - 100	0
pnv 358	9K/MB3/10-33/s	pPIC-9K	ND	80 - 100	0
pnv 359	9K/MB3/Tr/11-	pPIC-9K	ND	150 - 200	0
pnv 360	9K/MB3/Tr/11-	pPIC-9K	ND	150 - 200	0
pnv 361	9K/MB3/Tr/11-	pPIC-9K	ND	80 - 100	0
pnv 362	9K/MB3/Tr/11-	pPIC-9K	ND	80 - 100	0

Table 4. The expression of MB3 by recombinant clones with different expression cassettes. The main characteristic of the best clones.

CODE:	pnv318 s1/MB3/ 23/s	pnv322 D1/MB3/2-1/s	pnv345 9K/MB3/Tr/3-4/s	pnv350 9K/MB3/6-1/s
CHARACTERISTIC:				
Expression vector	pHIL-S1	pHIL-D2	pPIC 9K	pPIC 9K
Fused leader peptide	PHO1 (2.5kDa)	NO	a-factor(10kDa)	a-factor(10KDa)
Promoter for MB3	AOX1	AOX1	AOX1	AOX1
Size of expr. protein(s)	34.0; 37.5kDa	34.0kDa	43kDa	44kDa
Cleavage (Processing)	Cleavage (40-50%)	NO	NO	NO
Secretion	Weak, <10%	NO	NO	NO
MB3 degradation	<10%	<10%	<10%	<10%
Express level(mg/g)	2.0	3.0	2.0	1.5
Expression Level (mg/L)	300.0	600.0	150.0	150.0
Cytosol localization	60-70%	5-10%	50%	50%
Membrane association	30-40%	90-95%	50%	50%
Solubility	Partly soluble	Insoluble	Partly soluble	Partly soluble

Table 5. Codon Usage for *Pichia pastoris* and MB3

Pichia pastoris codon usage														
TTT	phe	F	11	TCT	ser	S	13	TAT	tyr	Y	6	TGT	cys	C	5
TTC	phe	F	5	TCC	ser	S	9	TAC	tyr	Y	8	TGC	cys	C	2
TTA	leu	L	3	TCA	ser	S	2	TAA	OCH	Z	-	TGA	OPA	Z	-
TTG	leu	L	26	TCG	ser	S	3	TAG	AMB	Z	-	TGG	trp	W	3
CCT	leu	L	4	CCT	pro	P	6	CAT	his	H	-	CTG	arg	R	4
CTC	leu	L	1	CCC	pro	P	5	CAC	his	H	3	CGC	arg	R	2
CTA	leu	L	4	CCA	pro	P	4	CAA	gln	Q	12	CGA	arg	R	-
CTG	leu	L	8	CCG	pro	P	1	CAG	gln	Q	1	CGG	arg	R	2
ATT	ile	I	8	ACT	thr	T	17	AAT	asn	N	9	AGT	ser	S	6
ATC	ile	I	7	ACC	thr	T	5	AAC	asn	N	4	AGC	ser	S	1
ATA	ile	I	3	ACA	thr	T	5	AAA	lys	K	15	AGA	arg	R	6
ATG	ile	M	4	ACG	thr	T	1	AAG	lys	K	14	AGG	arg	R	6
GTT	val	V	15	GCT	ala	A	17	GAT	asp	D	15	GGT	gly	G	13
GTC	val	V	6	GCC	ala	A	6	GAC	asp	D	12	GGC	gly	G	5
GTA	val	V	2	GCA	ala	A	9	GAA	glu	E	23	GGA	gly	G	6
GTG	val	V	10	GCG	ala	A	1	GAG	glu	E	11	GGG	gly	G	-

Outer membrane group B porin protein class 3 (MB3) codon usage														
TTT	phe	F	2	TCT	ser	S	8	TAT	tyr	Y	4	TGT	cys	C	-
TTC	phe	F	11	TCC	ser	S	7	TAC	tyr	Y	11	TGC	cys	C	-
TTA	leu	L	1	TCA	ser	S	-	TAA	OCH	Z	1	TGA	OPA	Z	-
TTG	leu	L	11	TCG	ser	S	4	TAG	AMB	Z	-	TGG	trp	W	4
CCT	leu	L	2	CCT	pro	P	2	CAT	his	H	2	CTG	arg	R	4
CTC	leu	L	3	CCC	pro	P	3	CAC	his	H	7	CGC	arg	R	8
CTA	leu	L	-	CCA	pro	P	-	CAA	gln	Q	10	CGA	arg	R	-
CTG	leu	L	7	CCG	pro	P	-	CAG	gln	Q	4	CGG	arg	R	1
ATT	ile	I	5	ACT	thr	T	5	AAT	asn	N	6	AGT	ser	S	-
ATC	ile	I	7	ACC	thr	T	7	AAC	asn	N	12	AGC	ser	S	9
ATA	ile	I	-	ACA	thr	T	-	AAA	lys	K	21	AGA	arg	R	1
ATG	met	M	2	ACG	thr	T	1	AAG	lys	K	2	AGG	arg	R	-
GTT	val	V	10	GCT	ala	A	4	GAT	asp	D	9	GGT	gly	G	14
GTC	val	V	5	GCC	ala	A	7	GAC	asp	D	12	GGC	gly	G	23
GTA	val	V	9	GCA	ala	A	9	GAA	glu	E	11	GGA	gly	G	1
GTG	val	V	7	GCG	ala	A	2	GAG	glu	E	4	GGG	gly	G	-

What Is Claimed Is:

1. A method for the high level expression of the outer membrane meningococcal group B porin protein or a fusion protein thereof in yeast, comprising:

(a) ligating into a plasmid having a selectable marker a gene coding for a protein selected from the group consisting of:

(i) a mature porin protein

(ii) a fusion protein which is a mature porin protein fused to a yeast secretion signal peptide;

wherein said gene is operably linked to a yeast promoter;

(b) transforming said plasmid containing said gene into a yeast strain;

(c) selecting the transformed yeast by growing said yeast in a culture medium allowing selection of said transformed yeast;

(d) growing the transformed yeast, and

(e) inducing expression of said protein to give yeast containing said protein;

wherein the protein so expressed comprises more than about 2% of the total protein expressed in said yeast.

2. The method according to claim 1, wherein the protein so expressed comprises about 3-5% of the total protein expressed in said yeast.

3. The method according to claim 1, wherein said mature porin protein is the *Neisseria meningitidis* mature outer membrane class 3 protein from serogroup B.

4. The method according to claim 1, wherein said yeast promoter is the AOX1 promoter.

5. The method according to claim 1, wherein said yeast secretion signal peptide is selected from the group consisting of the secretion signal of the *S. cerevisiae* α-mating factor prepro-peptide and the secretion signal of the *P. pastoris* acid phosphatase gene.

6. The method according to claim 1, wherein said plasmid is selected from the group consisting of pHIL-D2, pHIL-S1, pPIC9 and pPIC9K.

7. The method according to claim 1, wherein said gene comprises a nucleotide sequence that incorporates codons optimized for yeast codon usage.

8. The method according to claim 7, wherein said codons optimized for yeast codon usage are in the 5′ region of said gene.

9. The method according to claim 8, wherein said 5′ region of said gene is the nucleotide sequence:

5'-gac gtC acT Ttg tac ggT acT att aaG gcT ggT gtT gaG act tcc cgc tct gta ttt cac cag aac ggc caa gtt act gaa gtt aca-3'.

10. The method according to claim 8, wherein said yeast is *P. pastoris*.

11. The method of claim 1 wherein said yeast secretes said protein or fusion protein into a growth medium.

12. The method of claim 11 wherein said plasmid is selected from the group consisting of pHIL-S1, pPIC9, and pPIC9K.

13. A method of purifying the outer membrane meningococcal group B porin protein or fusion protein thereof obtained according to the method of claim 1 comprising:

(a) lysing said yeast obtained in step (d) to release said protein or fusion protein as an insoluble membrane bound fraction;

(b) washing said insoluble membrane bound fraction obtained in step (a) with a buffer to remove contaminating yeast cellular proteins;

(c) suspending and dissolving said insoluble membrane bound fraction obtained in step (b) in an aqueous solution of a denaturant;

(d) diluting the solution obtained in step (c) with a detergent; and

(e) purifying said protein or fusion protein by gel filtration and ion exchange chromatography.

14. A method of purifying the outer membrane meningococcal group B porin protein or fusion protein thereof obtained according to the method of claim 11 comprising:

(a) centrifuging said yeast culture which has expressed the protein to isolate the protein as soluble secreted material;

(b) removing contaminating yeast culture impurities from the soluble secreted material obtained in step (a) by precipitating said impurities with about 20% ethanol, wherein the soluble secreted material remains in the soluble fraction;

(c) precipitating the secreted material from the soluble fraction of step (b) with about 80% ethanol;

(d) washing the precipitated material obtained in step (c) with a buffer to remove contaminating yeast secreted proteins;

(e) suspending and dissolving the precipitated material obtained in step (d) in an aqueous solution of detergent; and

(f) purifying the protein by ion exchange chromatography.

15. A yeast host cell that contains a gene coding for a protein selected from the group consisting of:

(a) a mature porin protein

(b) a fusion protein which is a mature porin protein fused to a yeast secretion signal peptide.

16. The yeast host cell of claim 15, wherein said yeast contains more than one copy of said gene.

17. The yeast host cell of claim 15 wherein said mature porin protein is the *Neisseria meningitidis* mature outer membrane class 3 protein from serogroup B.

18. The yeast host cell of claim 17 wherein said plasmid is selected from the group consisting of pHIL-D2, pHIL-S1, pPIC9, pPIC9K and pAO815.

19. The yeast host cell of claim 15, wherein said yeast is *P. pastoris*.

20. The yeast host cell of claim 15, wherein the 5' region of the gene encoding said protein is encoded by the nucleotide sequence:

5'-gac gtC acT Ttg tac ggT acT att aaG gcT ggT gtT gaG act tcc cgc tct gta ttt cac cag aac ggc caa gtt act gaa gtt aca-3'.

21. A nucleotide sequence coding for an outer membrane meningococcal group B porin protein, wherein at least one codon has been changed to optimize yeast codon usage.

22. The nucleotide sequence of claim 21, wherein said porin protein is the mature outer membrane class 3 protein from serogroup B, and said codon changes are selected from the group of changes consisting of: (GTT to GTC at positions 4-6 of the native sequence), (ACC to ACT at positions 7-9 of the native sequence), (CTG to TTG at positions 10-12 of the native sequence), (GGC to GGT at positions 16-18 of the native sequence), (ACC to ACT at positions 19-21 of the native sequence), (ATC to ATT at positions 22-24 of the native sequence), (AAA to AAG at positions 25-27 of the native sequence), (GCC to GCT at positions 28-30 of the native sequence), (GGC to GGT at positions 31-33 of the native sequence), (GTA to GTT at positions 34-36 of the native sequence), (GAA to GAG at positions 37-39 of the native sequence);
wherein said positions are numbered from the first nucleotide of the native nucleotide sequence encoding said protein.

23. A vaccine comprising group A meningococcal polysaccharide (GAMP), group B meningococcal polysaccharide (GBMP), and group C meningococcal polysaccharide (GCMP) antigens, together with a pharmaceutically acceptable carrier.

24. The vaccine of claim 23, wherein said group A meningococcal polysaccharide (GAMP), group B meningococcal polysaccharide (GBMP), and

group C meningococcal polysaccharide (GCMP) antigens are each conjugated to a protein carrier.

25. The vaccine of claim 24, wherein said protein carrier to which said GBMP antigen is conjugated is class 3 *N. meningitidis* porin protein (PorB).

26. The vaccine of claim 24, wherein said protein carrier to which said GAMP antigen and said GCMP antigen are conjugated is tetanus toxoid.

27. The vaccine of claim 25, wherein said GBMP antigen is N-propionylated prior to being conjugated to PorB.

28. The vaccine of claim 24 wherein said vaccine comprises about 2 μg of the GAMP, GCMP and GBMP polysaccharide antigen conjugates.

29. The vaccine of claim 24, wherein said vaccine comprises about 5 μg of the GAMP, GCMP and GBMP polysaccharide antigen conjugates.

30. The vaccine of claim 24, wherein said vaccine comprises about 2 μg of the GAMP and GCMP polysaccharide antigen conjugates, and about 5 μg of the GBMP polysaccharide antigen conjugate.

31. A method of inducing an immune response in a mammal, comprising administering a vaccine comprising group A meningococcal polysaccharide (GAMP), group B meningococcal polysaccharide (GBMP), and group C meningococcal polysaccharide (GCMP) antigens, together with a pharmaceutically acceptable carrier, in an amount sufficient to induce an immune response in a mammal.

32. The method of claim 31, wherein said group A meningococcal polysaccharide (GAMP), group B meningococcal polysaccharide (GBMP), and group C meningococcal polysaccharide (GCMP) antigens are each conjugated to a protein carrier.

33. The method of claim 31, wherein said mammal is a human.

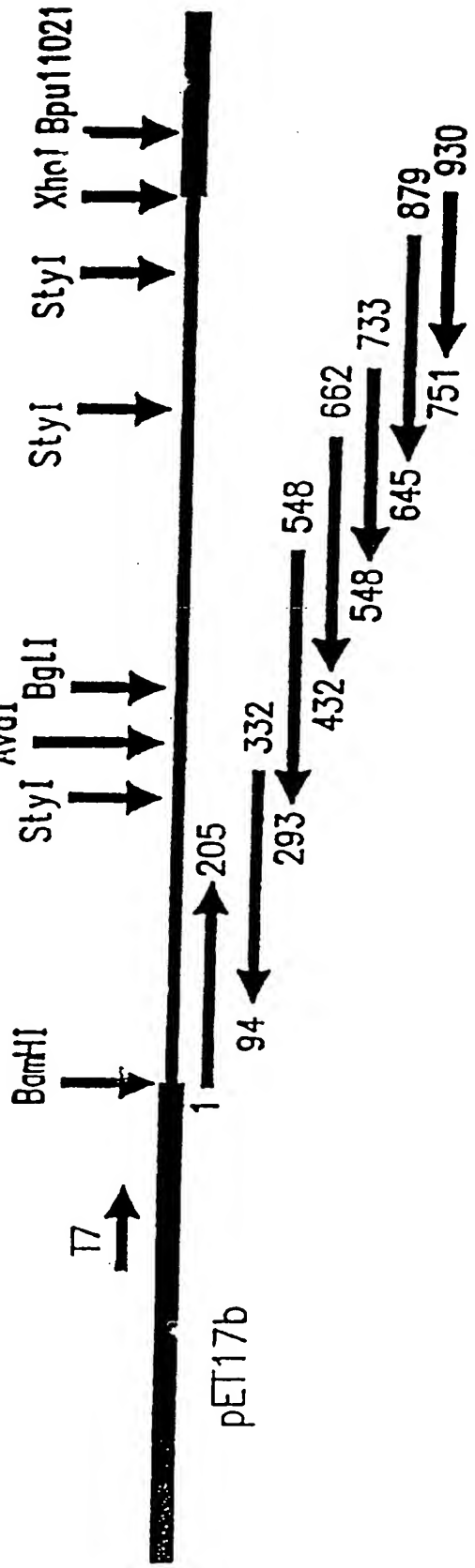


FIG. 1

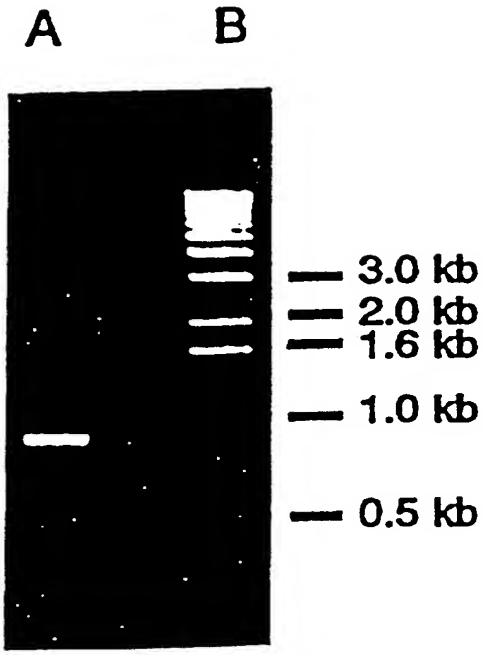


FIG. 2

FIG. 3A

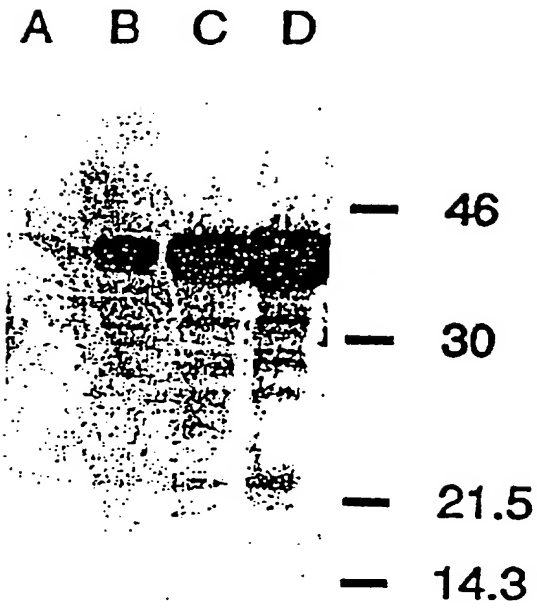


FIG. 3B

```
        10 C  C   20        30        40        50        60        70
TTGTACGGTACAATTAAAGCAGGCGTAGAAACTTCCCGCTCTGTATTTCACCAGAACGGCCAAGTTACTG
AACATGCCATGTTAATTTCGTCCGCATCTTTGAAGGGCGAGACATAAAGTGGTCTTGCCGGTTCAATGAC
 L  Y  G  T  I  K  A  G  V  E  T  S  R  S  V  F  H  Q  N  G  Q  V  T

        80        90       100       110       120       130       140
AAGTTACAACCGCTACCGGCATCGTTGATTTGGGTTCGAAAATCGGCTTCAAAGGCCAAGAAGACCTCGG
TTCAATGTTGGCGATGGCCGTAGCAACTAAACCCAAGCTTTTAGCCGAAGTTTCCGGTTCTTCTGGAGCC
E  V  T  T  A  T  G  I  V  D  L  G  S  K  I  G  F  K  G  Q  E  D  L  G

       150       160       170       180       190       200       210
TAACGGCCTGAAAGCCATTTGGCAGGTTGAGCAAAAAGCATCTATCGCCGGTACTGACTCCGGTTGGGGC
ATTGCCGGACTTTCGGTAAACCGTCCAACTCGTTTTTCGTAGATAGCGGCCATGACTGAGGCCAACCCCG
  N  G  L  K  A  I  W  Q  V  E  Q  K  A  S  I  A  G  T  D  S  G  W  G

       220       230       240       250       260       270       280
AACCGCCAATCCTTCATCGGCTTGAAAGGCGGCTTCGGTAAATTGCGCGTCGGTCGTTTGAACAGCGTCC
TTGGCGGTTAGGAAGTAGCCGAACTTTCCGCCGAAGCCATTTAACGCGCAGCCAGCAAACTTGTCGCAGG
 N  R  Q  S  F  I  G  L  K  G  G  F  G  K  L  R  V  G  R  L  N  S  V

       290       300       310       320       330       340       350
TGAAAGACACCGGCGACATCAATCCTTGGGATAGCAAAAGCGACTATTTGGGTGTAAACAAAATTGCCGA
ACTTTCTGTGGCCGCTGTAGTTAGGAACCCTATCGTTTTCGCTGATAAACCCACATTTGTTTTAACGGCT
L  K  D  T  G  D  I  N  P  W  D  S  K  S  D  Y  L  G  V  N  K  I  A  E

       360       370       380       390       400       410       420
ACCCGAGGCACGCCTCATTTCCGTACGCTACGATTCTCCCGAATTTGCCGGCCTCAGCGGCAGCGTACAA
TGGGCTCCGTGCGGAGTAAAGGCATGCGATGCTAAGAGGGCTTAAACGGCCGGAGTCGCCGTCGCATGTT
  P  E  A  R  L  I  S  V  R  Y  D  S  P  E  F  A  G  L  S  G  S  V  Q

       430       440       450       460       470       480       490
TACGCGCTTAACGACAATGCAGGCAGACATAACAGCGAATCTTACCACGCCGGCTTCAACTACAAAAACG
ATGCGCGAATTGCTGTTACGTCCGTCTGTATTGTCGCTTAGAATGGTGCGGCCGAAGTTGATGTTTTTGC
 Y  A  L  N  D  N  A  G  R  H  N  S  E  S  Y  H  A  G  F  N  Y  K  N

       500       510       520       530       540       550       560
GTGGCTTCTTCGTGCAATATGGCGGTGCCTATAAAAGACATCATCAAGTGCAAGAGGGCTTGAATATTGA
CACCGAAGAAGCACGTTATACCGCCACGGATATTTTCTGTAGTAGTTCACGTTCTCCCGAACTTATAACT
G  G  F  F  V  Q  Y  G  G  A  Y  K  R  H  H  Q  V  Q  E  G  L  N  I  E

       570       580       590       600       610       620       630
GAAATACCAGATTCACCGTTTGGTCAGCGGTTACGACAATGATGCCCTGTACGCTTCCGTAGCCGTACAG
CTTTATGGTCTAAGTGGCAAACCAGTCGCCAATGCTGTTACTACGGGACATGCGAAGGCATCGGCATGTC
  K  Y  Q  I  H  R  L  V  S  G  Y  D  N  D  A  L  Y  A  S  V  A  V  Q

       640       650       660       670       680       690       700
CAACAAGACGCGAAACTGACTGATGCTTCCAATTCGCACAACTCTCAAACCGAAGTTGCCGCTACCTTGG
GTTGTTCTGCGCTTTGACTGACTACGAAGGTTAAGCGTGTTGAGAGTTTGGCTTCAACGGCGATGGAACC
 Q  Q  D  A  K  L  T  D  A  S  N  S  H  N  S  Q  T  E  V  A  A  T  L

       710       720       730       740       750       760       770
CATACCGCTTCGGCAACGTAACGCCCCGAGTTTCTTACGCCCACGGCTTCAAAGGTTTGGTTGATGATGC
GTATGGCGAAGCCGTTGCATTGCGGGGCTCAAAGAATGCGGGTGCCGAAGTTTCCAAACCAACTACTACG
A  Y  R  F  G  N  V  T  P  R  V  S  Y  A  H  G  F  K  G  L  V  D  D  A

       780       790       800       810       820       830       840
AGACATAGGCAACGAATACGACCAAGTGGTTGTCGGTGCGGAATACGACTTCTCCAAACGCACTTCTGCC
TCTGTATCCGTTGCTTATGCTGGTTCACCAACAGCCACGCCTTATGCTGAAGAGGTTTGCGTGAAGACGG
  D  I  G  N  E  Y  D  Q  V  V  V  G  A  E  Y  D  F  S  K  R  T  S  A
       850       860       870       880       890       900       910
TTGGTTTCTGCCGGTTGGTTGCAAGAAGGCAAAGGCGAAAACAAATTCGTAGCGACTGCCGGCGGTGTTG
AACCAAAGACGGCCAACCAACGTTCTTCCGTTTCCGCTTTTGTTTAAGCATCGCTGACGGCCGCCACAAC
 L  V  S  A  G  W  L  Q  E  G  K  G  E  N  K  F  V  A  T  A  G  G  V

       920       930
GTCTGCGTCACAAATTCTAA
CAGACGCAGTGTTTAAGATT
G  L  R  H  K  F
```

Fig. 4

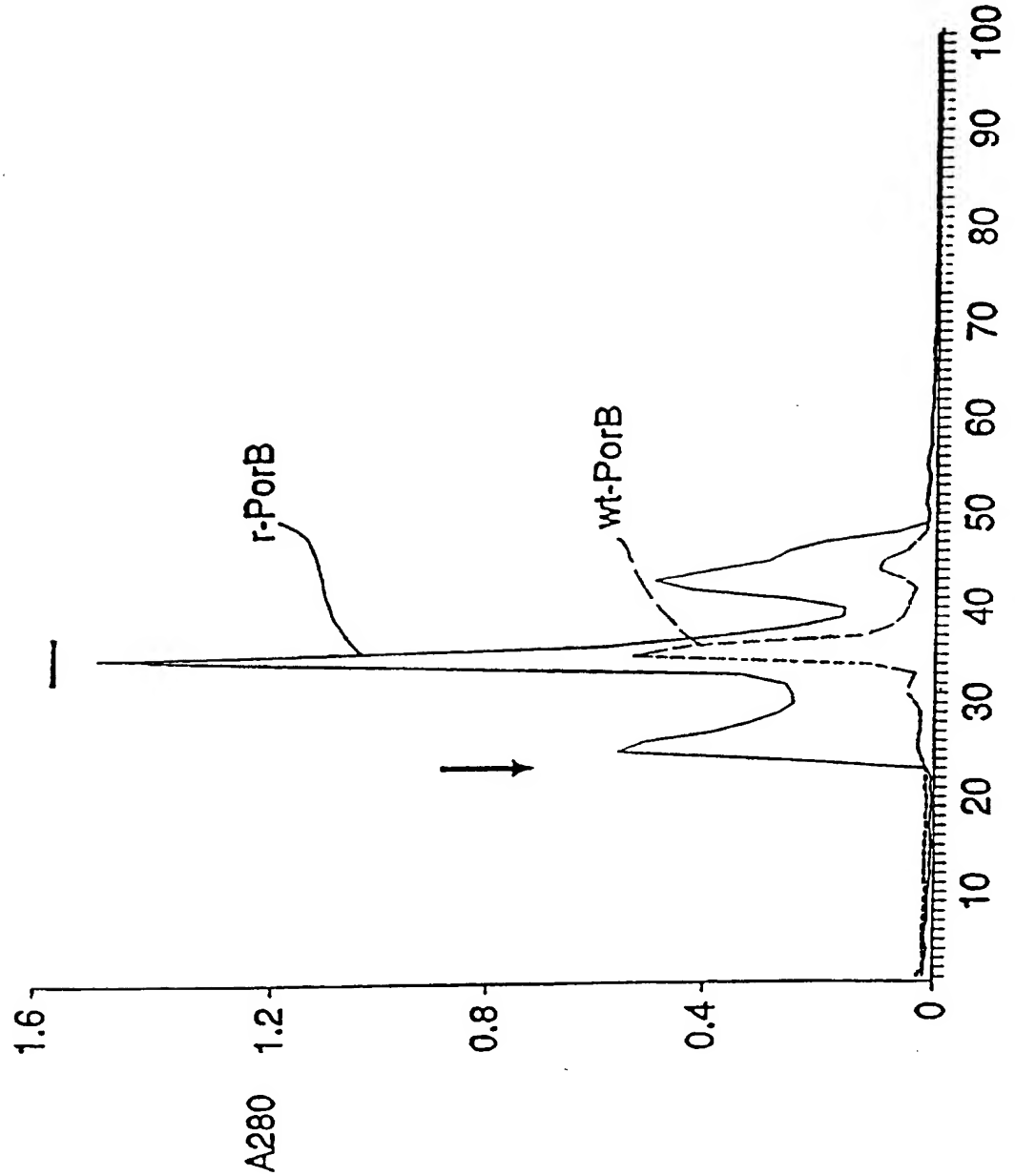


FIG.5

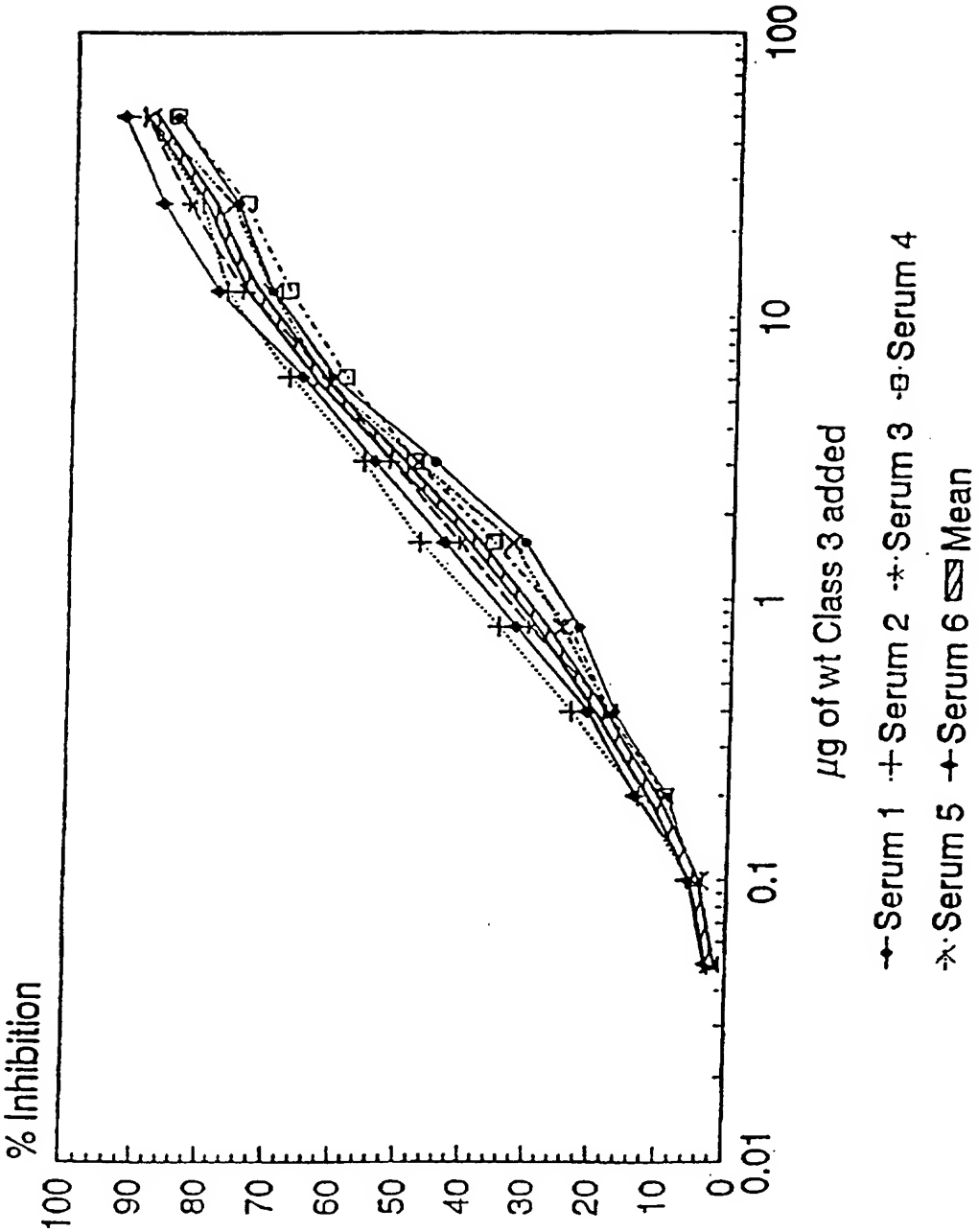


FIG.6

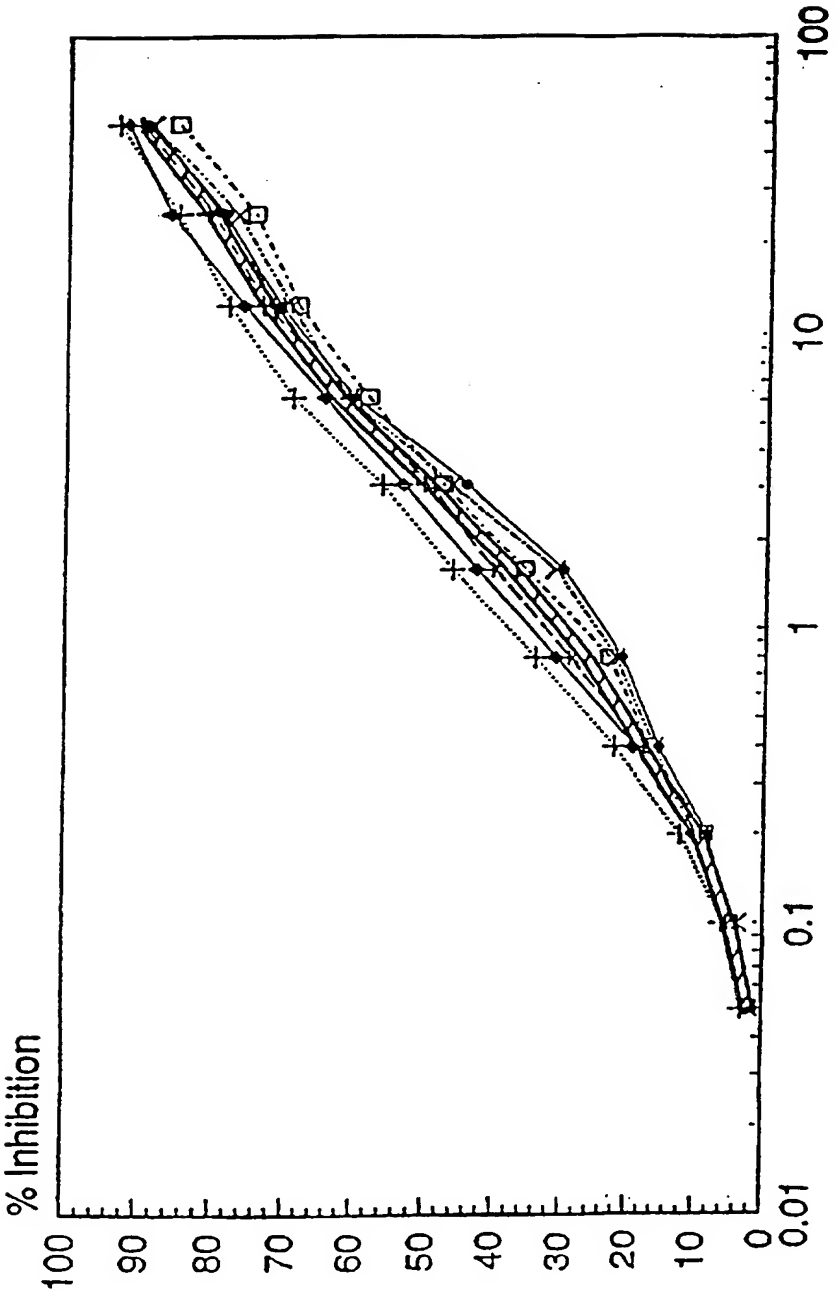


FIG. 7

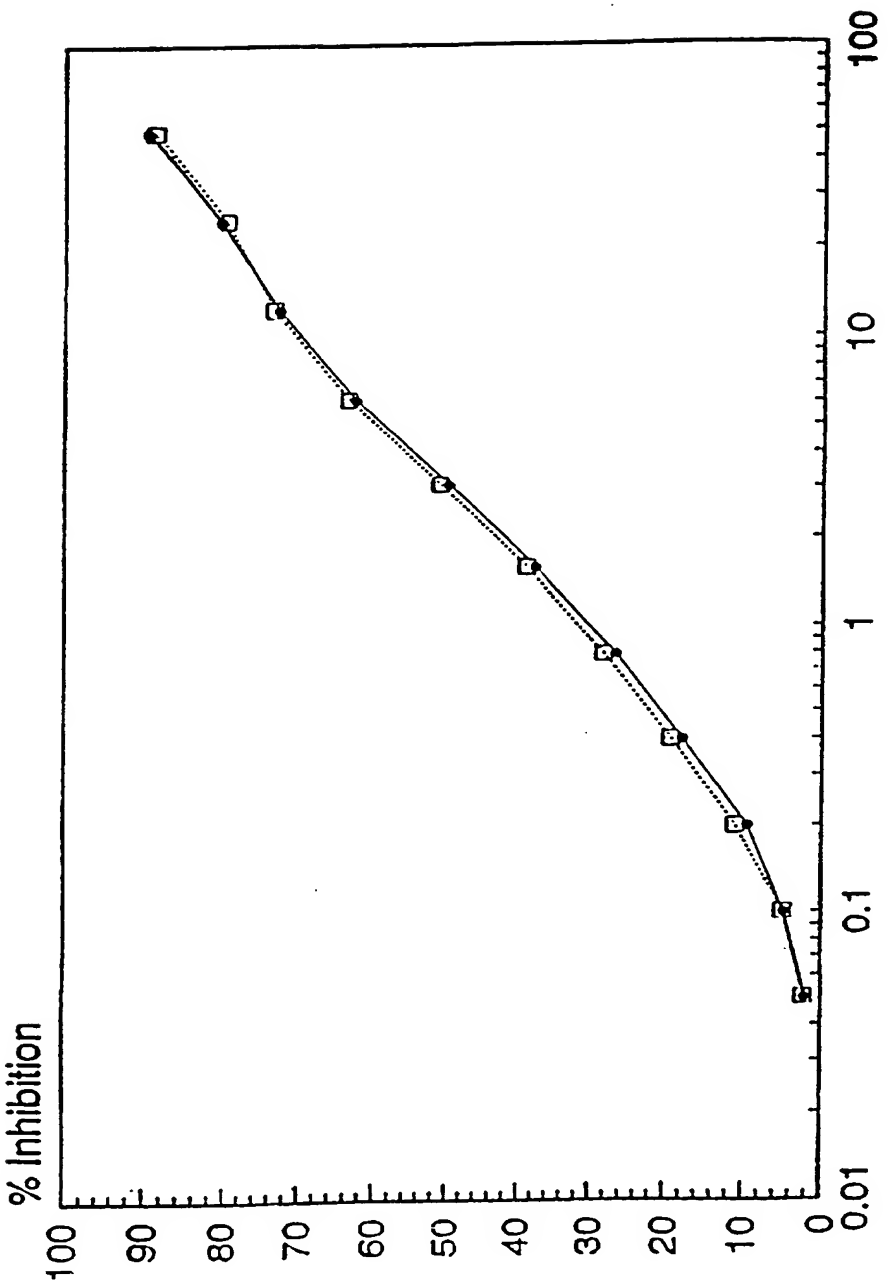


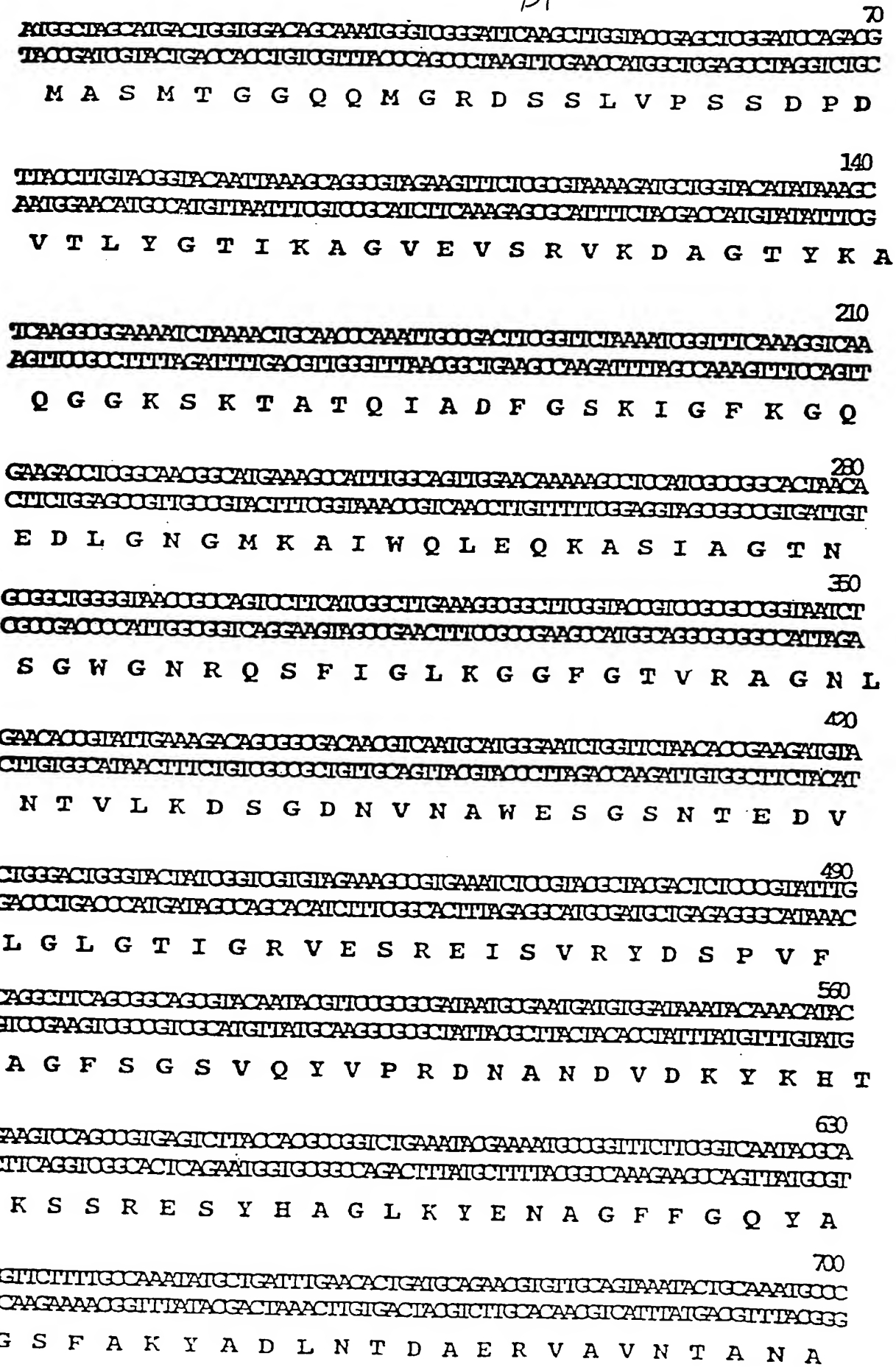
FIG. 8

```
ATGGACGTTACCTTGTACGGTACAATTAAAGCAGGCGTAGAAGTTTCTCGCGTAAAAGATGCTGGTACAT 70
TACCTGCAATGGAACATGCCATGTTAATTTCGTCCGCATCTTCAAAGAGCGCATTTTCTACGACCATGTA
 M  D  V  T  L  Y  G  T  I  K  A  G  V  E  V  S  R  V  K  D  A  G  T

ATAAAGCTCAAGGCGGAAAATCTAAAACTGCAACCCAAATTGCCGACTTCGGTTCTAAAATCGGTTTCAA 140
TATTTCGAGTTCCGCCTTTTAGATTTTGACGTTGGGTTTAACGGCTGAAGCCAAGATTTTAGCCAAAGTT
 Y  K  A  Q  G  G  K  S  K  T  A  T  Q  I  A  D  F  G  S  K  I  G  F  K

AGGTCAAGAAGACCTCGGCAACGGCATGAAAGCCATTTGGCAGTTGGAACAAAAAGCCTCCATCGCCGGC 210
TCCAGTTCTTCTGGAGCCGTTGCCGTACTTTCGGTAAACCGTCAACCTTGTTTTTCGGAGGTAGCGGCCG
  G  Q  E  D  L  G  N  G  M  K  A  I  W  Q  L  E  Q  K  A  S  I  A  G

ACTAACAGCGGCTGGGGTAACCGCCAGTCCTTCATCGGCTTGAAAGGCGGCTTCGGTACCGTCCGCGCCG 280
TGATTGTCGCCGACCCCATTGGCGGTCAGGAAGTAGCCGAACTTTCCGCCGAAGCCATGGCAGGCGCGGC
 T  N  S  G  W  G  N  R  Q  S  F  I  G  L  K  G  G  F  G  T  V  R  A

GTAATCTGAACACCGTATTGAAAGACAGCGGCGACAACGTCAATGCATGGGAATCTGGTTCTAACACCGA 350
CATTAGACTTGTGGCATAACTTTCTGTCGCCGCTGTTGCAGTTACGTACCCTTAGACCAAGATTGTGGCT
 G  N  L  N  T  V  L  K  D  S  G  D  N  V  N  A  W  E  S  G  S  N  T  E

AGATGTACTGGGACTGGGTACTATCGGTCGTGTAGAAAGCCGTGAAATCTCCGTACGCTACGACTCTCCC 420
TCTACATGACCCTGACCCATGATAGCCAGCACATCTTTCGGCACTTTAGAGGCATGCGATGCTGAGAGGG
  D  V  L  G  L  G  T  I  G  R  V  E  S  R  E  I  S  V  R  Y  D  S  P

GTATTTGCAGGCTTCAGCGGCAGCGTACAATACGTTCCGCGCGATAATGCGAATGATGTGGATAAATACA 490
CATAAACGTCCGAAGTCGCCGTCGCATGTTATGCAAGGCGCGCTATTACGCTTACTACACCTATTTATGT
 V  F  A  G  F  S  G  S  V  Q  Y  V  P  R  D  N  A  N  D  V  D  K  Y

AACATACGAAGTCCAGCCGTGAGTCTTACCACGCCGGTCTGAAATACGAAAATGCCGGTTTCTTCGGTCA 560
TTGTATGCTTCAGGTCGGCACTCAGAATGGTGCGGCCAGACTTTATGCTTTTACGGCCAAAGAAGCCAGT
 K  H  T  K  S  S  R  E  S  Y  H  A  G  L  K  Y  E  N  A  G  F  F  G  Q

ATACGCAGGTTCTTTTGCCAAATATGCTGATTTGAACACTGATGCAGAACGTGTTGCAGTAAATACTGCA 630
TATGCGTCCAAGAAAACGGTTTATACGACTAAACTTGTGACTACGTCTTGCACAACGTCATTTATGACGT
  Y  A  G  S  F  A  K  Y  A  D  L  N  T  D  A  E  R  V  A  V  N  T  A

AATGCCCATCCTGTTAAGGATTACCAAGTACACCGCGTAGTTGCCGGTTACGATGCCAATGACCTGTACG 700
TTACGGGTAGGACAATTCCTAATGGTTCATGTGGCGCATCAACGGCCAATGCTACGGTTACTGGACATGC
 N  A  H  P  V  K  D  Y  Q  V  H  R  V  V  A  G  Y  D  A  N  D  L  Y

TTTCTGTTGCCGGTCAGTATGAAGCTGCTAAAAACAACGAGGTTGGTTCTACCAAGGGTAAAAAACACGA 770
AAAGACAACGGCCAGTCATACTTCGACGATTTTTGTTGCTCCAACCAAGATGGTTCCCATTTTTTGTGCT
 V  S  V  A  G  Q  Y  E  A  A  K  N  N  E  V  G  S  T  K  G  K  K  H  E

GCAAACTCAAGTTGCCGCTACTGCCGCTTACCGTTTTGGCAACGTAACGCCTCGCGTTTCTTACGCCCAC 840
CGTTTGAGTTCAACGGCGATGACGGCGAATGGCAAAACCGTTGCATTGCGGAGCGCAAAGAATGCGGGTG
  Q  T  Q  V  A  A  T  A  A  Y  R  F  G  N  V  T  P  R  V  S  Y  A  H
```

Fig. 9A

```
GGCTTCAAAGCTAAAGTGAATGGCGTGAAAGACGCAAATTACCAATACGACCAAGTTATCGTTGGTGCCG 910
CCGAAGTTTCGATTTCACTTACCGCACTTTCTGCGTTTAATGGTTATGCTGGTTCAATAGCAACCACGGC
 G  F  K  A  K  V  N  G  V  K  D  A  N  Y  Q  Y  D  Q  V  I  V  G  A

ACTACGACTTCTCCAAACGCACTTCCGCTCTGGTTTCTGCCGGTTGGTTGAAACAAGGTAAAGGCGCGGG 980
TGATGCTGAAGAGGTTTGCGTGAAGGCGAGACCAAAGACGGCCAACCAACTTTGTTCCATTTCCGCGCCC
 D  Y  D  F  S  K  R  T  S  A  L  V  S  A  G  W  L  K  Q  G  K  G  A  G

AAAAGTCGAACAAACTGCCAGCATGGTTGGTCTGCGTCACAAATTCTAA 1029
TTTTCAGCTTGTTTGACGGTCGTACCAACCAGACGCAGTGTTTAAGATT
 K  V  E  Q  T  A  S  M  V  G  L  R  H  K  F
```

Fig. 9B



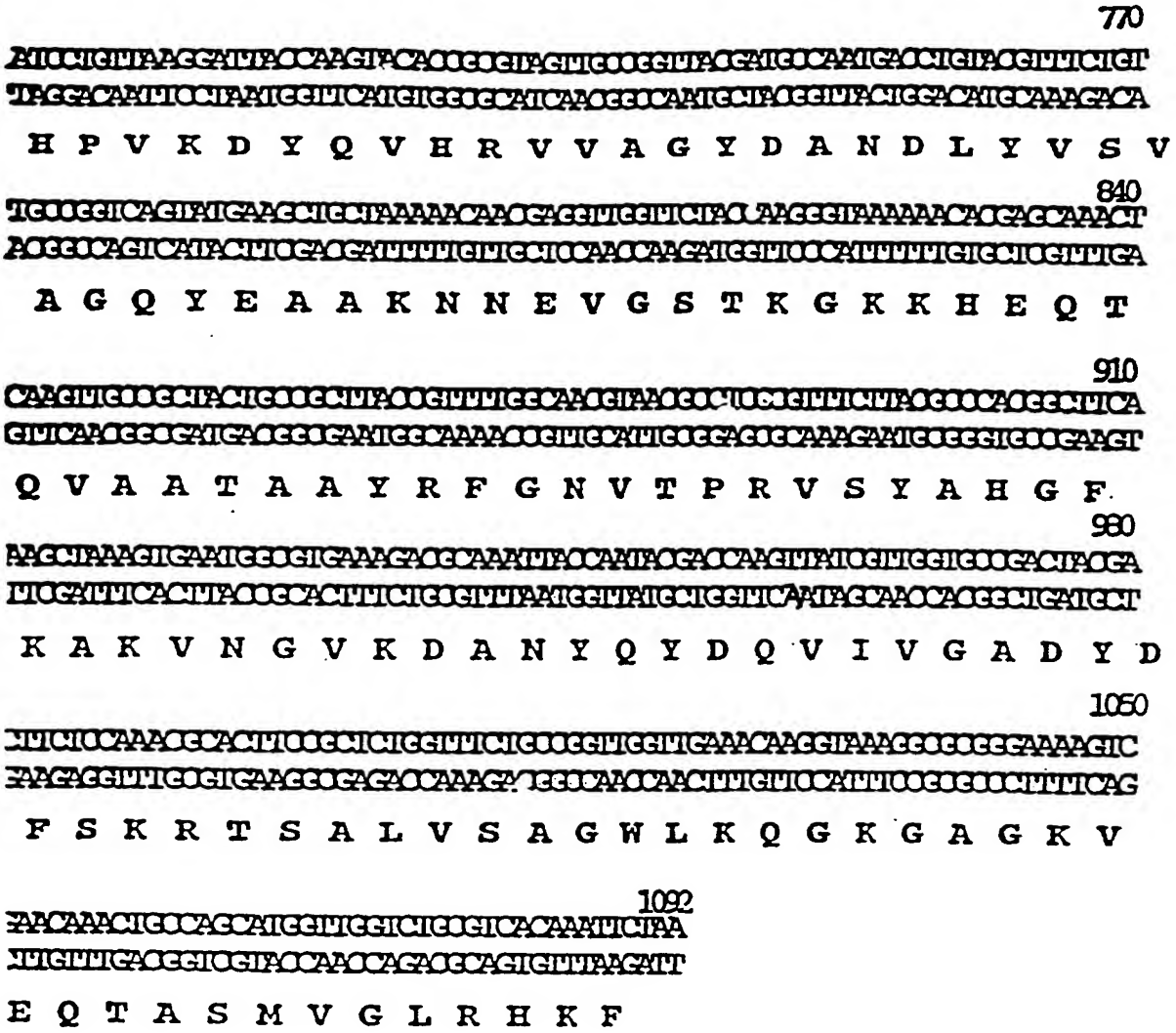


Fig. 10B

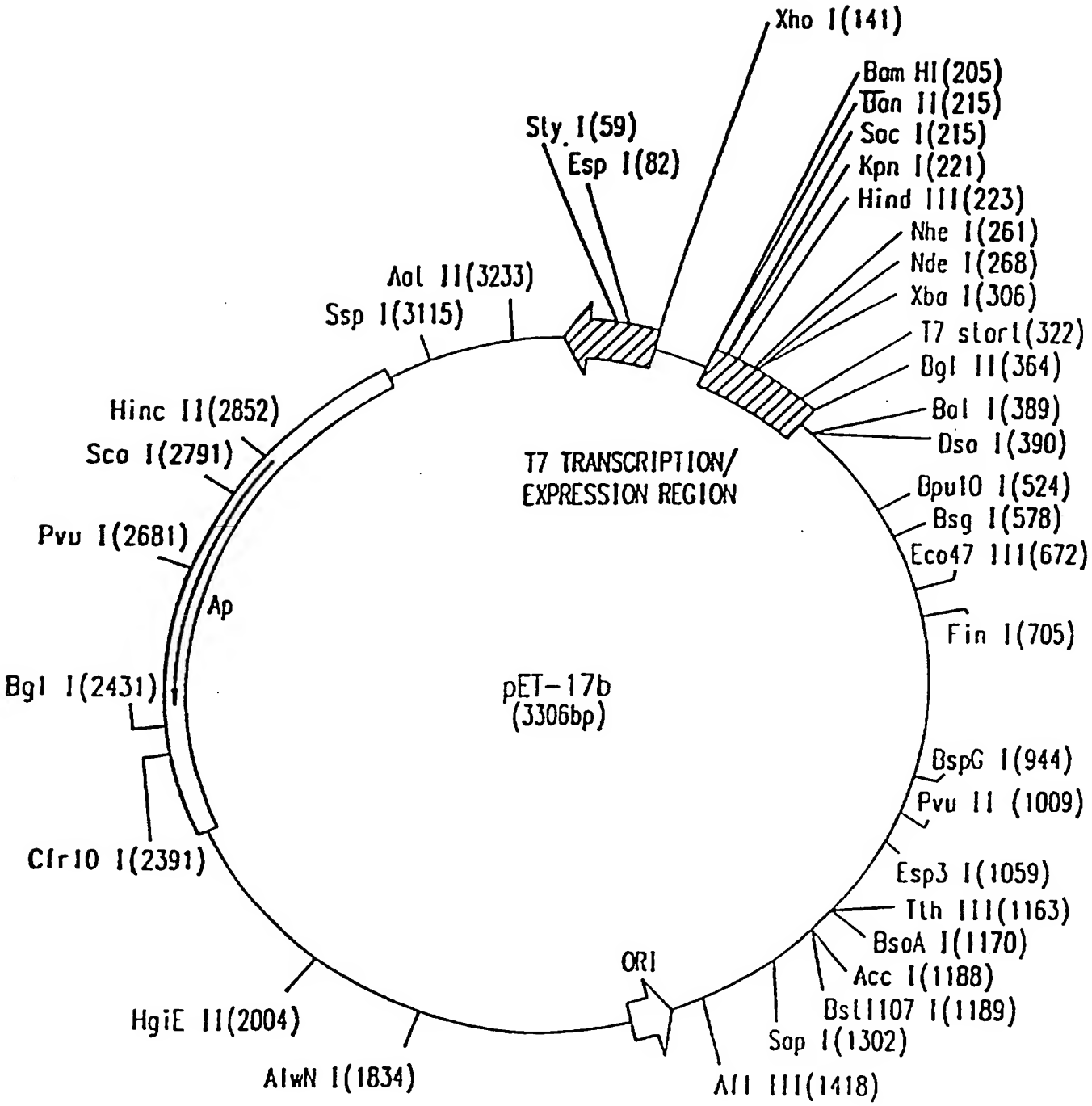
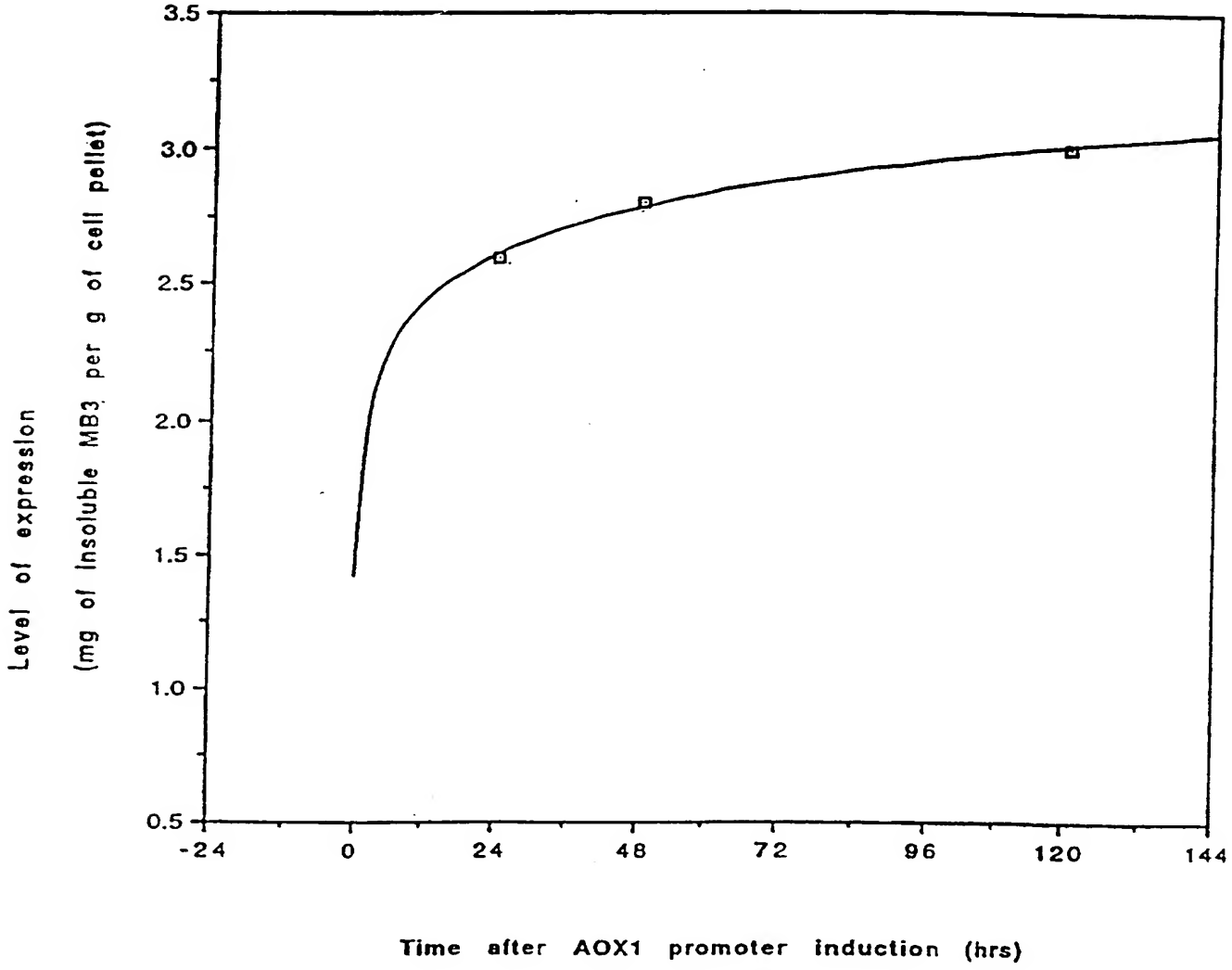


FIG.11A

```
                                                                  XbaI
 SgfI
AGATCTCGAT CCCGCGAAAT TAATACGACT CACTATAGGG AGACCACAAC GGTTTCCCTC      60

                                        NcoI   NheI
TAGAAATAAT TTTGTTTAAC TTAAAGAAGG AGATATACAT ATG GCT AGC ATG ACT       115
                                            Met Ala Ser Met Thr
                                             1              5

                                    HindIII KpnI  SacI  (BamHI)
GGT GGA CAG CAA ATG GGT CGG GAT TCA AGC TTG GTA CCG AGC TCG GAT       163
Gly Gly Gln Gln Met Gly Arg Asp Ser Ser Leu Val Pro Ser Ser Asp
                10                  15                  20

                                                                     187
CTG CAG GTT ACC TTG TAC GGT ACA
Leu Gln Val Thr Leu Tyr Gly Thr
            25

                                                  XbaI
GTT GGT CTG CGT CAC AAA TTC TAACTCGAGC AGATCCGGCT GCTAACAAAG          51
Val Gly Leu Arg His Lys Phe
  1             5

                                                                     54
CCC
```

FIG.11B

Fig. ~~10~~ 12 The production levels of the expressed MB3 (clone: pnv 322; expression vector: pHIL-D2)



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DNA Strider 1.0 ### Wednesday, January 17, 1996 8:26:14 PM

pNV15

~~MB3/pnv15~~/pET24A -> 1-phase Translation

DNA sequence 942 b.p. ATGgacgttacc ... cacaaattctaa linear

```
1   /   1                                   31  /  11
ATG gac gtt acc ctg tac ggc acc att aaa gca ggc gta gaa act tcc cgc tct gta ttt
met asp val thr leu tyr gly thr ile lys ala gly val glu thr ser arg ser val phe
61  /  21                                  91  /  31
cac cag aac ggc caa gtt act gaa gtt aca acc gct acc ggc atc gtt gat ttg ggt tcg
his gln asn gly gln val thr glu val thr thr ala thr gly ile val asp leu gly ser
121 /  41                                  151 /  51
aaa atc ggc ttc aaa ggc caa gaa gac ctc ggt aac ggc ctg aaa gcc att tgg cag gtt
lys ile gly phe lys gly gln glu asp leu gly asn gly leu lys ala ile trp gln val
181 /  61                                  211 /  71
gag caa aaa gca tct atc gcc ggt act gac tcc ggt tgg ggc aac cgc caa tcc ttc atc
glu gln lys ala ser ile ala gly thr asp ser gly trp gly asn arg gln ser phe ile
241 /  81                                  271 /  91
ggc ttg aaa ggc ggc ttc ggt aaa ttg cgc gtc ggt cgt ttg aac agc gtc ctg aaa gac
gly leu lys gly gly phe gly lys leu arg val gly arg leu asn ser val leu lys asp
301 /  101                                 331 /  111
acc ggc gac atc aat cct tgg gat agc aaa agc gac tat ttg ggt gta aac aaa att gcc
thr gly asp ile asn pro trp asp ser lys ser asp tyr leu gly val asn lys ile ala
361 /  121                                 391 /  131
gaa ccc gag gca cgc ctc att tcc gta cgc tac gat tct ccc gaa ttt gcc ggc ctc agc
glu pro glu ala arg leu ile ser val arg tyr asp ser pro glu phe ala gly leu ser
421 /  141                                 451 /  151
ggc agc gta caa tac gcg ctt aac gac aat gca ggc aga cat aac agc gaa tct tac cac
gly ser val gln tyr ala leu asn asp asn ala gly arg his asn ser glu ser tyr his
481 /  161                                 511 /  171
gcc ggc ttc aac tac aaa aac ggt ggc ttc ttc gtg caa tat ggc ggt gcc tat aaa aga
ala gly phe asn tyr lys asn gly gly phe phe val gln tyr gly gly ala tyr lys arg
541 /  181                                 571 /  191
cat cat caa gtg caa gag ggc ttg aat att gag aaa tac cag att cac cgt ttg gtc agc
his his gln val gln glu gly leu asn ile glu lys tyr gln ile his arg leu val ser
601 /  201                                 631 /  211
ggt tac gac aat gat gcc ctg tac gct tcc gta gcc gta cag caa caa gac gcg aaa ctg
gly tyr asp asn asp ala leu tyr ala ser val ala val gln gln gln asp ala lys leu
661 /  221                                 691 /  231
act gat gct tcc aat tcg cac aac tct caa acc gaa gtt gcc gct acc ttg gca tac cgc
thr asp ala ser asn ser his asn ser gln thr glu val ala ala thr leu ala tyr arg
721 /  241                                 751 /  251
ttc ggc aac gta acg ccc cga gtt tct tac gcc cac ggc ttc aaa ggt ttg gtt gat gat
phe gly asn val thr pro arg val ser tyr ala his gly phe lys gly leu val asp asp
781 /  261                                 811 /  271
gca gac ata ggc aac gaa tac gac caa gtg gtt gtc ggt gcg gaa tac gac ttc tcc aaa
ala asp ile gly asn glu tyr asp gln val val val gly ala glu tyr asp phe ser lys
841 /  281                                 871 /  291
cgc act tct gcc ttg gtt tct gcc ggt tgg ttg caa gaa ggc aaa ggc gaa aac aaa ttc
arg thr ser ala leu val ser ala gly trp leu gln glu gly lys gly glu asn lys phe
901 /  301                                 931 /  311
gta gcg act gcc ggc ggt gtc ggt ctg cgc cac aaa ttc taa
val ala thr ala gly gly val gly leu arg his lys phe ~~ocr~~
```

coding seq of MB3

DNA Strider 1.0 ### Wednesday, January 17, 1996 8:17:35 PM

Men.Class3 opt. -> 1-phase Translation

DNA sequence 942 b.p. ATGgacgtCacT ... cacaaattctaa linear

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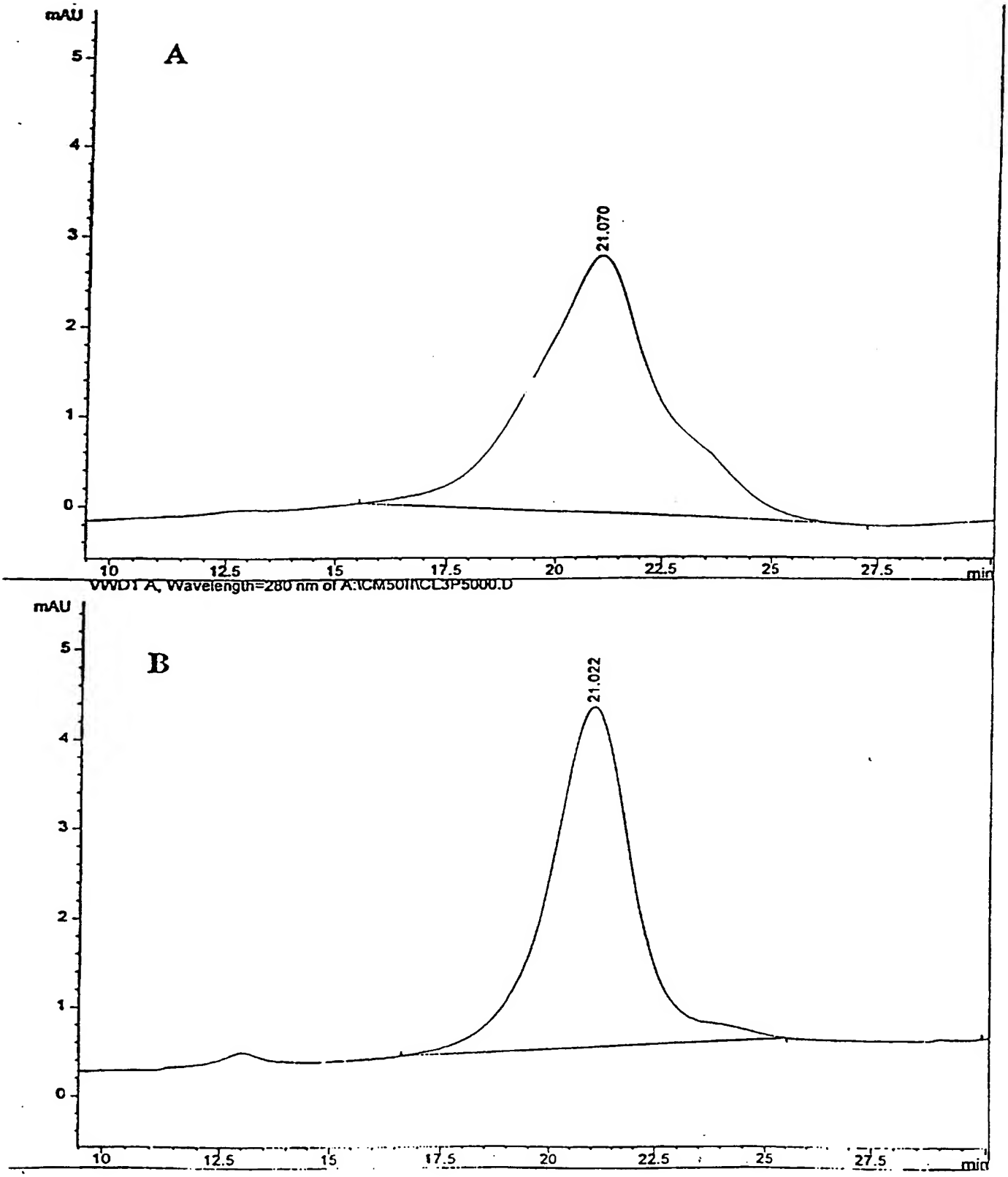
1    /    1                                    31   /   11
ATG gac gtC acT Ttg tac ggT acT att aaG gcT ggT gtT gaG act tcc cgc tct gta ttt
met asp val thr leu tyr gly thr ile lys ala gly val glu thr ser arg ser val phe
61   /   21                                    91   /   31
cac cag aac ggc caa gtt act gaa gtt aca acc gct acc ggc atc gtt gat ttg ggt tcg
his gln asn gly gln val thr glu val thr thr ala thr gly ile val asp leu gly ser
121  /   41                                    151  /   51
aaa atc ggc ttc aaa ggc caa gaa gac ctc ggt aac ggc ctg aaa gcc att tgg cag gtt
lys ile gly phe lys gly gln glu asp leu gly asn gly leu lys ala ile trp gln val
181  /   61                                    211  /   71
gag caa aaa gca tct atc gcc ggt act gac tcc ggt tgg ggc aac cgc caa tcc ttc atc
glu gln lys ala ser ile ala gly thr asp ser gly trp gly asn arg gln ser phe ile
241  /   81                                    271  /   91
ggc ttg aaa ggc ggc ttc ggt aaa ttg cgc gtc ggt cgt ttg aac agc gtc ctg aaa gac
gly leu lys gly gly phe gly lys leu arg val gly arg leu asn ser val leu lys asp
301  /  101                                    331  /  111
acc ggc gac atc aat cct tgg gat agc aaa agc gac tat ttg ggt gta aac aaa att gcc
thr gly asp ile asn pro trp asp ser lys ser asp tyr leu gly val asn lys ile ala
361  /  121                                    391  /  131
gaa ccc gag gca cgc ctc att tcc gta cgc tac gat tct ccc gaa ttt gcc ggc ctc agc
glu pro glu ala arg leu ile ser val arg tyr asp ser pro glu phe ala gly leu ser
421  /  141                                    451  /  151
ggc agc gta caa tac gcg ctt aac gac aat gca ggc aga cat aac agc gaa tct tac cac
gly ser val gln tyr ala leu asn asp asn ala gly arg his asn ser glu ser tyr his
481  /  161                                    511  /  171
gcc ggc ttc aac tac aaa aac ggt ggc ttc ttc gtg caa tat ggc ggt gcc tat aaa aga
ala gly phe asn tyr lys asn gly gly phe phe val gln tyr gly gly ala tyr lys arg
541  /  181                                    571  /  191
cat cat caa gtg caa gag ggc ttg aat att gag aaa tac cag att cac cgt ttg gtc agc
his his gln val gln glu gly leu asn ile glu lys tyr gln ile his arg leu val ser
601  /  201                                    631  /  211
ggt tac gac aat gat gcc ctg tac gct tcc gta gcc gta cag caa caa gac gcg aaa ctg
gly tyr asp asn asp ala leu tyr ala ser val ala val gln gln gln asp ala lys leu
661  /  221                                    691  /  231
act gat gct tcc aat tcg cac aac tct caa acc gaa gtt gcc gct acc ttg gca tac cgc
thr asp ala ser asn ser his asn ser gln thr glu val ala ala thr leu ala tyr arg
721  /  241                                    751  /  251
ttc ggc aac gta acg ccc cga gtt tct tac gcc cac ggc ttc aaa ggt ttg gtt gat gat
phe gly asn val thr pro arg val ser tyr ala his gly phe lys gly leu val asp asp
781  /  261                                    811  /  271
gca gac ata ggc aac gaa tac gac caa gtg gtt gtc ggt gcg gaa tac gac ttc tcc aaa
ala asp ile gly asn glu tyr asp gln val val val gly ala glu tyr asp phe ser lys
841  /  281                                    871  /  291
cgc act tct gcc ttg gtt tct gcc ggt tgg ttg caa gaa ggc aaa ggc gaa aac aaa ttc
arg thr ser ala leu val ser ala gly trp leu gln glu gly lys gly glu asn lys phe
901  /  301                                    931  /  311
gta gcg act gcc ggc ggt gtc ggt ctg cgc cac aaa ttc taa
val ala thr ala gly gly val gly leu arg his lys phe ~~OCH~~
```

FIGURE 14



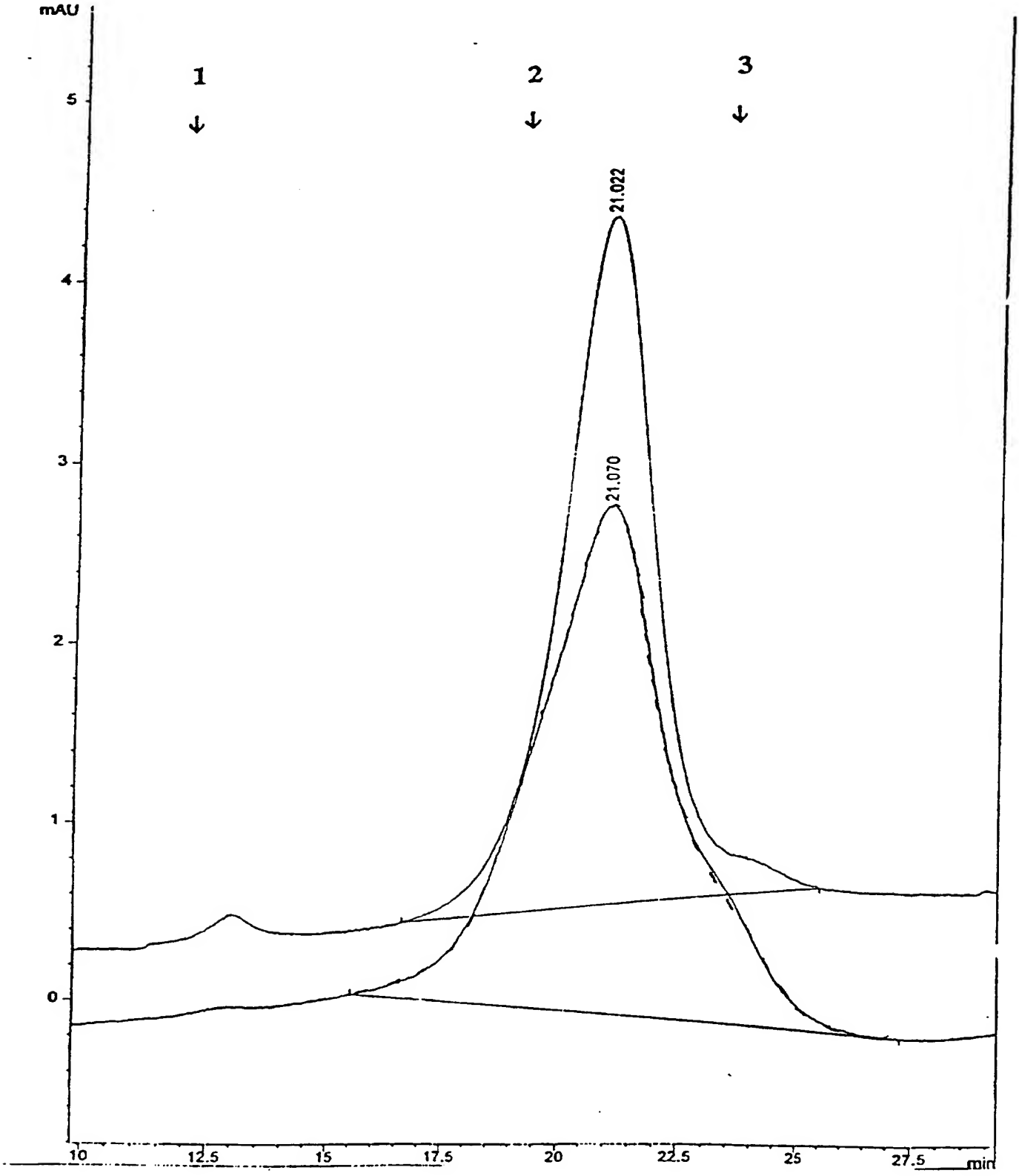


FIGURE 15

Fig. 16A

DNA Strider 1.0 Wednesday, January 17, 1996 8:59:15 PM

III-D2/MB3/21—> List (pnv 322)

A sequence 9156 b.p. AGATCgcggccg ... CGCGCGAGGCAG linear

```
         |  10        |  20        |  30        |  40        |  50        |  60
   1 AGATCgcggc cgcgatctaa catccaaaga CGAAAGGTTG AATGAAACCT TTTTGCCATC  60
  61 CGACATCCAC AGGTCCATTC TCACACATAA GTGCCAAACG CAACAGGAGG GGATACACTA 120
 121 GCAGCAGACC GTTGCAAACG CAGGACCTCC ACTCCTCTTC TCCTCAACAC CCACTTTTGC 180
 181 CATCGAAAAA CCAGCCCAGT TATTGGGCTT GATTGGAGCT CGCTCATTCC AATTCCTTCT 240
 241 ATTAGGCTAC TAACACCATG ACTTTATTAG CCTGTCTATC CTGCCCCCCC TGCCGAGGTC 300
 301 ATGTTTGTTT ATTTCCGAAT GCAACAAGCT CCGCATTACA CCCGAACATC ACTCCAGATG 360
 361 AGGGCTTTCT GAGTGTGGGG TCAAATAGTT TCATGTTCCC AAATGGCCCA AAACTGACAG 420
 421 TTTAAACGCT GTCTTGGAAC CTAATATGAC AAAAGCGTGA TCTCATCCAA GATGAACTAA 480
 481 CTTTGGTTCG TTGAAATGCT AACGGCCAGT TGGTCAAAAA GAAACTTCCA AAAGTCGCCA 540
 541 TACCGTTTGT CTTGTTTGGT ATTGATTGAC GAATGCTCAA AAATAATCTC ATTAATGCTT 600
 601 AGCGCAGTCT CTCTATCGCT TCTGAACCCG GTGGCACCTG TGCCGAAACG CAAATGGGGA 660
 661 AACAACCCGC TTTTTGGATG ATTATGCATT GTCCTCCACA TTGTATGCTT CCAAGATTCT 720
 721 GGTGGGAATA CTGCTGATAG CCTAACGTTC ATGATCAAAA TTTAACTGTT CTAACCCCTA 780
 781 CTTGACAGCC AATATATAAA CAGAAGGAAG CTGCCCTGTC TTAAACCTTT TTTTTTATCA 840
 841 TCATTATTAG CTTACTTTCA TAATTGCGAC TGGTTCCAAT TGACAAGCTT TTGATTTTAA 900
 901 CGACTTTTAA CGACAACTTG AGAAGATCAA AAAACAACTA ATTATTCGAA ACGAGGAATT 960
 961 CATGgacgtc actttgtacg gtactattaa ggctggtgtt gagacttccc gctctgtatt 1020
1021 tcaccagaac ggccaagtta ctgaagttac aaccgctacc ggcatcgttg atttgggttc 1080
1081 gaaaatcggc ttcaaaggcc aagaagacct cggtaacggc ctgaaagcca tttggcaggt 1140
1141 tgagcaaaaa gcatctatcg ccggtactga ctccggttgg ggcaaccgcc aatccttcat 1200
1201 cggcttgaaa ggcggcttcg gtaaattgcg cgtcggtcgt ttgaacagcg tcctgaaaga 1260
1261 caccggcgac atcaatcctt gggatagcaa aagcgactat ttgggtgtaa acaaaattgc 1320
1321 cgaacccgag gcacgcctca tttccgtacg ctacgattct cccgaatttg ccggcctcag 1380
1381 cggcagcgta caatacgcgc ttaacgacaa tgcaggcaga cataacagcg aatcttacca 1440
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1861 cgtagcgact gccggcggtg ttggtctgcg ccacaaattc taaGAATTCC CTTAGACATC 1920
1921 ACTGTTCCTC AGTTCAAGTT GGGCATTACG AGAAGACCGG TCTTGCTAGA TTCTAATCAA 1980
1981 GAGGATGTCA GAATGCCATT TGCCTGAGAG ATGCAGGCTT CATTTTTGAT ACTTTTTTAT 2040
2041 TTGTAACCTA TATAGTATAG GATTTTTTTT GTCATTTTGT TTCTTCTCGT ACGAGCTTGC 2100
2101 TCCTGATCAG CCTATCTCGC AGCTGATGAA TATCTTGTGG TAGGGGTTTG GGAAAATCAT 2160
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2221 AGAAGTTCGT TTGTGCAAGC TTATCGATAA GCTTTAATGC GGTAGTTTAT CACAGTTAAA 2280
2281 TTGCTAACGC AGTCAGGCAC CGTGTATGAA ATCTAACAAT GCGCTCATCG TCATCCTCGG 2340
2341 CACCGTCACC CTGGATGCTG TAGGCATAGG CTTGGTTATG CCGGTACTGC CGGGCCTCTT 2400
2401 GCGGGATATC GTCCATTCCG ACAGCATCGC CAGTCACTAT GGCGTGCTGC TAGCGCTATA 2460
2461 TGCGTTGATG CAATTTCTAT GCGCACCCGT TCTCGGAGCA CTGTCCGACC GCTTTGGCCG 2520
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2581 CACACCCGTC CTGTGGATCT ATCGAATCTA AATGTAAGTT AAAATCTCTA AATAATTAAA 2640
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2701 CAGCAGCCAG ATCCATCACT GCTTGGCCAA TATGTTTCAG TCCCTCAGGA GTTACGTCTT 2760
2761 GTGAAGTGAT GAACTTCTGG AAGGTTGCAG TGTTAACTCC GCTGTATTGA CGGGCATATC 2820
2821 CGTACGTTGG CAAAGTGTGG TTGGTACCGG AGGAGTAATC TCCACAACTC TCTGGAGAGT 2880
2881 AGGCACCAAC AAACACAGAT CCAGCGTGTT GTACTTGATC AACATAAGAA GAAGCATTCT 2940
2941 CGATTTGCAG GATCAAGTGT TCAGGAGCGT ACTGATTGGA CATTTCCAAA GCCTGCTCGT 3000
3001 AGGTTGCAAC CGATAGGGTT GTAGAGTGTG CAATACACTT GCGTACAATT TCAACCCTTG 3060
3061 GCAACTGCAC AGCTTGGTTG TGAACAGCAT CTTCAATTCT GGCAAGCTCC TTGTCTGTCA 3120
3121 TATCGACAGC CAACAGAATC ACCTGGGAAT CAATACCATG TTCAGCTTGA GACAGAAGGT 3180
3181 CTGAGGCAAC GAAATCTGGA TCAGCGTATT TATCAGCAAT AACTAGAACT TCAGAAGGCC 3240
3241 CAGCAGGCAT GTCAATACTA CACAGGGCTG ATGTGTCATT TTGAACCATC ATCTTGGCAG 3300
3301 CAGTAACGAA CTGGTTTCCT GGACCAAATA TTTTGTCACA CTTAGGAACA GTTTCTGTTC 3360
3361 CGTAAGCCAT AGCAGCTACT GCCTGGGCGC CTCCTGCTAG CACGATACAC TTAGCACCAA 3420
3421 CCTTGTGGGC AACGTAGATG ACTTCTGGGG TAAGGGTACC ATCCTTCTTA CGTGGAGATG 3480
3481 CAAAAACAAT TTCTTTGCAA CCAGCAACTT TGGCAGGAAC ACCCAGCATC AGGGAAGTGG 3540
3541 AAGGCAGAAT TGCGGTTCCA CCAGGAATAT AGAGGCCAAC TTTCTCAATA GGTCTTGCAA 3600
3601 AACGAGAGCA GACTACACCA GGGCAAGTCT CAACTTGCAA CGTCTCCGTT AGTTGAGCTT 3660
3661 CATGGAATTT CCTGACGTTA TCTATAGAGA GATCAATGGC TCTCTTAACG TTATCTGGCA 3720
3721 ATTGCATAAG TTCCTCTGGG AAAGGACGTT CTAACACAGG TGTCTTCAAA GCGACTCCAT 3780
3781 CAAACTTGGC AGTTAGTTCT AAAAGGGCTT TGTCACCATT TTGACGAACA TTGTCGACAA 3840
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3961 AAGCGACTTC TTTAGGTTTG GATTCTTCTT TAGGTTGTTC CTTGGTGTAT CCTGGCTTGG 4020
4021 CATCTCCTTT CCTTCTAGTG ACCTTTAGGC ACTTCATATC CAGGTTTCTC TCCACCTCGT 4080
4081 CCAACGTCAC ACCGTACTTG GCACATCTAA CTAATGCAAA ATAAAATAAG TCAGCACATT 4140
4141 CCCAGGCTAT ATCTTCCTTG GATTTAGCTT CTGCAAGTTC ATCAGCTTCC TCCCTAATTT 4200
4201 TAGCGTTCAA CAAAACTTCG TCGTCAAATA ACCGTTTGGT ATAAGAACCT TCTGGAGCAT 4260
4261 TGCTCTTACG ATCCCACAAG GTGGCTTCCA TGGCTCTAAG ACCCTTTGAT TGGCCAAAAC 4320
4321 AGGAAGTGCG TTCCAAGTGA CAGAAACCAA CACCTGTTTG TTCAACCACA AATTTCAAGC 4380
4381 AGTCTCCATC ACAATCCAAT TCGATACCCA GCAACTTTTG AGTTGCTCCA GATGTAGCAC 4440
4441 CTTTATACCA CAAACCGTGA CGACGAGATT GGTAGACTCC AGTTTGTGTC CTTATAGCCT 4500
4501 CCGGAATAGA CTTTTTGGAC GAGTACACCA GGCCCAACGA GTAATTAGAA GAGTCAGCCA 4560
4561 CCAAAGTAGT GAATAGACCA TCGGGCCCGT CAGTAGTCAA AGACGCCAAC AAAATTTCAC 4620
4621 TGACAGGGAA CTTTTTGACA TCTTCAGAAA GTTCGTATTC AGTAGTCAAT TGCCGAGCAT 4680
4681 CAATAATGGG GATTATACCA GAAGCAACAG TGGAAGTCAC ATCTACCAAC TTTGCGGTCT 4740
4741 CAGAAAAAGC ATAAACAGTT CTACTACCGC CATTAGTGAA ACTTTTCAAA TCGCCCAGTG 4800
4801 GAGAAGAAAA AGGCACAGCG ATACTAGCAT TAGCGGGCAA GGATGCAACT TTATCAACCA 4860
4861 GGGTCCTATA GATAACCCTA GCGCCTGGGA TCATCCTTTG GACAACTCTT TCTGCCAAAT 4920
4921 CTAGGTCCAA AATCACTTCA TTGATACCAT TATTGTACAA CTTGAGCAAG TTGTCGATCA 4980
4981 GCTCCTCAAA TTGGTCCTCT GTAACGGATG ACTCAACTTG CACATTAACT TGAAGCTCAG 5040
5041 TCGATTGAGT GAACTTGATC AGGTTGTGCA GCTGGTCAGC AGCATAGGGA AACACGGCTT 5100
5101 TTCCTACCAA ACTCAAGGAA TTATCAAACT CTGCAACACT TGCGTATGCA GGTAGCAAGC 5160
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5221 TTTATTATCA GTGAGTCAGT CATCAGGAGA TCCTCTACGC CGGACGCATC GTGGCCGGCA 5280
5281 TCACCGGCGC CACAGGTGCG GTTGCTGGCG CCTATATCGC CGACATCACC GATGGGGAAG 5340
5341 ATCGGGCTCG CCACTTCGGG CTCATGAGCG CTTGTTTCGG CGTGGGTATG GTGGCAGGCC 5400
5401 CCGTGGCCGG GGGACTGTTG GGCGCCATCT CCTTGCATGC ACCATTCCTT GCGGCGGCGG 5460
5461 TGCTCAACGG CCTCAACCTA CTACTGGGCT GCTTCCTAAT GCAGGAGTCG CATAAGGGAG 5520
5521 AGCGTCGAGT ATCTATGATT GGAAGTATGG GAATGGTGAT ACCCGCATTC TTCAGTGTCT 5580
5581 TGAGGTCTCC TATCAGATTA TGCCCAACTA AAGCAACCGG AGGAGGAGAT TTCATGGTAA 5640
5641 ATTTCTCTGA CTTTTGGTCA TCAGTAGACT CGAACTGTGA GACTATCTCG GTTATGACAG 5700
5701 CAGAAATGTC CTTCTTGGAG ACAGTAAATG AAGTCCCACC AATAAAGAAA TCCTTGTTAT 5760
5761 CAGGAACAAA CTTCTTGTTT CGAACTTTTT CGGTGCCTTG AACTATAAAA TGTAGAGTGG 5820
5821 ATATGTCGGG TAGGAATGGA GCGGGCAAAT GCTTACCTTC TGGACCTTCA AGAGGTATGT 5880
5881 AGGGTTTGTA GATACTGATC CCAACTTCAG TGACAACGTT GCTATTTCGT TCAAACCATT 5940
5941 CCGAATCCAG AGAAATCAAA GTTGTTTGTC TACTATTGAT CCAAGCCAGT GCGGTCTTGA 6000
6001 AACTGACAAT AGTGTGCTCG TGTTTTGAGG TCATCTTTGT ATGAATAAAT CTAGTCTTTG 6060
6061 ATCTAAATAA TCTTGACGAG CCAAGGCGAT AAATACCCAA ATCTAAAACT CTTTTAAAAC 6120
6121 GTTAAAAGGA CAAGTATGTC TGCCTGTATT AAACCCCAAA TCAGCTCGTA GTCTGATCCT 6180
6181 CATCAACTTG AGGGGCACTA TCTTGTTTTA GAGAAATTTG CGGAGATGCG ATATCGAGAA 6240
6241 AAAGGTACGC TGATTTTAAA CGTGAAATTT ATCTCAAGAT CgcggccGCG ATCTCGAATA 6300
6301 ATAACTGTTA TTTTTCAGTG TTCCCGATCT GCGTCTATTT CACAATACCA ACATGAGTCA 6360
6361 GCTTATCGAT GATAAGCTGT CAAACATGAG AATTAATTCG ATGATAAGCT GTCAAACATG 6420
6421 AGAAATCTTG AAGACGAAAG GGCCTCGTGA TACGCCTATT TTTATAGGTT AATGTCATGA 6480
6481 TAATAATGGT TTCTTAGACG TCAGGTGGCA CTTTTCGGGG AAATGTGCGC GGAACCCCTA 6540
6541 TTTGTTTATT TTTCTAAATA CATTCAAATA TGTATCCGCT CATGAGACAA TAACCCTGAT 6600
6601 AAATGCTTCA ATAATATTGA AAAAGGAAGA GTATGAGTAT TCAACATTTC CGTGTCGCCC 6660
6661 TTATTCCCTT TTTTGCGGCA TTTTGCCTTC CTGTTTTTGC TCACCCAGAA ACGCTGGTGA 6720
6721 AAGTAAAAGA TGCTGAAGAT CAGTTGGGTG CACGAGTGGG TTACATCGAA CTGGATCTCA 6780
6781 ACAGCGGTAA GATCCTTGAG AGTTTTCGCC CCGAAGAACG TTTTCCAATG ATGAGCACTT 6840
6841 TTAAAGTTCT GCTATGTGGC GCGGTATTAT CCCGTGTTGA CGCCGGGCAA GAGCAACTCG 6900
6901 GTCGCCGCAT ACACTATTCT CAGAATGACT TGGTTGAGTA CTCACCAGTC ACAGAAAAGC 6960
6961 ATCTTACGGA TGGCATGACA GTAAGAGAAT TATGCAGTGC TGCCATAACC ATGAGTGATA 7020
7021 ACACTGCGGC CAACTTACTT CTGACAACGA TCGGAGGACC GAAGGAGCTA ACCGCTTTTT 7080
7081 TGCACAACAT GGGGGATCAT GTAACTCGCC TTGATCGTTG GGAACCGGAG CTGAATGAAG 7140
7141 CCATACCAAA CGACGAGCGT GACACCACGA TGCCTGCAGC AATGGCAACA ACGTTGCGCA 7200
7201 AACTATTAAC TGGCGAACTA CTTACTCTAG CTTCCCGGCA ACAATTAATA GACTGGATGG 7260
7261 AGGCGGATAA AGTTGCAGGA CCACTTCTGC GCTCGGCCCT TCCGGCTGGC TGGTTTATTG 7320
7321 CTGATAAATC TGGAGCCGGT GAGCGTGGGT CTCGCGGTAT CATTGCAGCA CTGGGGCCAG 7380
7381 ATGGTAAGCC CTCCCGTATC GTAGTTATCT ACACGACGGG GAGTCAGGCA ACTATGGATG 7440
7441 AACGAAATAG ACAGATCGCT GAGATAGGTG CCTCACTGAT TAAGCATTGG TAACTGTCAG 7500
7501 ACCAAGTTTA CTCATATATA CTTTAGATTG ATTTAAATTG TAAACGTTAA TATTTTGTTA 7560
7561 AAATTCGCGT TAAATTTTTG TTAAATCAGC TCATTTTTTA ACCAATAGGC CGAAATCGGC 7620
7621 AAAATCCCTT ATAAATCAAA AGAATAGACC GAGATAGGGT TGAGTGTTGT TCCAGTTTGG 7680
7681 AACAAGAGTC CACTATTAAA GAACGTGGAC TCCAACGTCA AAGGGCGAAA AACCGTCTAT 7740
7741 CAGGGCGATG GCCCACTACG TGAACCATCA CCCTAATCAA GTTTTTTGCG GTCGAGGTGC 7800
7801 CGTAAAGCAC TAAATCGGAA CCCTAAAGGG AGCCCCCGAT TTAGAGCTTG ACGGGGAAAG 7860
7861 CCGGCGAACG TGGCGAGAAA GGAAGGGAAG AAAGCGAAAG GAGCGGGCGC TAGGGCGCTG 7920
7921 GCAAGTGTAG CGGTCACGCT GCGCGTAACC ACCACACCCG CCGCGCTTAA TGCGCCGCTA 7980
7981 CAGGGCGCGT AAAAGGATCT AGGTGAAGAT CCTTTTTGAT AATCTCATGA CCAAAATCCC 8040
8041 TTAACGTGAG TTTTCGTTCC ACTGAGCGTC AGACCCCGTA GAAAAGATCA AAGGATCTTC 8100
8101 TTGAGATCCT TTTTTTCTGC GCGTAATCTG CTGCTTGCAA ACAAAAAAAC CACCGCTACC 8160
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FIG 16C

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8161 AGCGGTGGTT TGTTTGCCGG ATCAA[redacted]  ACCAACTCTT TTTCCGAAGG TAACTGGCTT 82[redacted]
8221 CAGCAGAGCG CAGATACCAA ATACTGTCCT  TCTAGTGTAG CCGTAGTTAG GCCACCACTT 8280
8281 CAAGAACTCT GTAGCACCGC CTACATACCT  CGCTCTGCTA ATCCTGTTAC CAGTGGCTGC 8340
8341 TGCCAGTGGC GATAAGTCGT GTCTTACCGG  GTTGGACTCA AGACGATAGT TACCGGATAA 8400
8401 GGCGCAGCGG TCGGGCTGAA CGGGGGGTTC  GTGCACACAG CCCAGCTTGG AGCGAACGAC 8460
8461 CTACACCGAA CTGAGATACC TACAGCGTGA  GCATTGAGAA AGCGCCACGC TTCCCGAAGG 8520
8521 GAGAAAGGCG GACAGGTATC CGGTAAGCGG  CAGGGTCGGA ACAGGAGAGC GCACGAGGGA 8580
8581 GCTTCCAGGG GGAAACGCCT GGTATCTTTA  TAGTCCTGTC GGGTTTCGCC ACCTCTGACT 8640
8641 TGAGCGTCGA TTTTTGTGAT GCTCGTCAGG  GGGGCGGAGC CTATGGAAAA ACGCCAGCAA 8700
8701 CGCGGCCTTT TTACGGTTCC TGGCCTTTTG  CTGGCCTTTT GCTCACATGT TCTTTCCTGC 8760
8761 GTTATCCCCT GATTCTGTGG ATAACCGTAT  TACCGCCTTT GAGTGAGCTG ATACCGCTCG 8820
8821 CCGCAGCCGA ACGACCGAGC GCAGCGAGTC  AGTGAGCGAG GAAGCGGAAG AGCGCCTGAT 8880
8881 GCGGTATTTT CTCCTTACGC ATCTGTGCGG  TATTTCACAC CGCATATGGT GCACTCTCAG 8940
8941 TACAATCTGC TCTGATGCCG CATAGTTAAG  CCAGTATACA CTCCGCTATC GCTACGTGAC 9000
9001 TGGGTCATGG CTGCGCCCCG ACACCCGCCA  ACACCCGCTG ACGCGCCCTG ACGGGCTTGT 9060
9061 CTGCTCCCGG CATCCGCTTA CAGACAAGCT  GTGACCGTCT CCGGGAGCTG CATGTGTCAG 9120
9121 AGGTTTTCAC CGTCATCACC GAAACGCGCG  AGGCAG                          9156
          |  10       |  20       |  30       |  40       |  50       |  60
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Fig 17.

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PHIL-S1/MB3/23 -> List (pnv 318)

DNA sequence 9191 b.p. AGATCTAACATC ... AGTTATTATTCG linear

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 121 TGCAAACGCA GGACCTCCAC TCCTCTTCTC CTCAACACCC ACTTTTGCCA TCGAAAAACC 180
 181 AGCCCAGTTA TTGGGCTTGA TTGGAGCTCG CTCATTCCAA TTCCTTCTAT TAGGCTACTA 240
 241 ACACCATGAC TTTATTAGCC TGTCTATCCT GGCCCCCCTG GCGAGGTCAT GTTTGTTTAT 300
 301 TTCCGAATGC AACAAGCTCC GCATTACACC CGAACATCAC TCCAGATGAG GGCTTTCTGA 360
 361 GTGTGGGGTC AAATAGTTTC ATGTTCCCAA ATGGCCCAAA ACTGACAGTT TAAACGCTGT 420
 421 CTTGGAACCT AATATGACAA AAGCGTGATC TCATCCAAGA TGAACTAAGT TTGGTTCGTT 480
 481 GAAATGCTAA CGGCCAGTTG GTCAAAAAGA AACTTCCAAA AGTCGCCATA CCGTTTGTCT 540
 541 TGTTTGGTAT TGATTGACGA ATGCTCAAAA ATAATCTCAT TAATGCTTAG CGCAGTCTCT 600
 601 CTATCGCTTC TGAACCCGGT GGCACCTGTG CCGAAACGCA AATGGGGAAA CAACCCGCTT 660
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 781 TATATAAACA GAAGGAAGCT GCCCTGTCTT AAACCTTTTT TTTTATCATC ATTATTAGCT 840
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2101 AGTATAGGAT TTTTTTTGTC ATTTTGTTTC TTCTCGTACG AGCTTGCTCC TGATCAGCCT 2160
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2281 TGCAAGCTTA TCGATAAGCT TTAATGCGGT AGTTTATCAC AGTTAAATTG CTAACGCAGT 2340
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3661 TACACCAGGG CAAGTCTCAA CTTGCAACGT CTCCGTTAGT TGAGCTTCAT GGAATTTCCT 3720
3721 GACGTTATCT ATAGAGAGAT CAATGGCTCT CTTAACGTTA TCTGGCAATT GCATAAGTTC 3780
3781 CTCTCGGAAA GGAGCTTCTA ACACAGGTGT CTTCAAAGCC ACTCCATCAA ACTTGGCAGT 3840
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3961 GGAGGCCTTA GAAACGTCAA TTTTGCACAA TTCAATACGA CCTTCAGAAG GGACTTCTTT 4020
4021 AGGTTTGGAT TCTTCTTTAG GTTGTTCCTT GGTGTATCCT GGCTTGGCAT CTCCTTTCCT 4080
4081 TCTAGTGACC TTTAGGGACT TCATATCCAG GTTTCTCTCC ACCTCGTCCA ACGTCACACC 4140
4141 GTACTTGCCA CATCTAACTA ATGCAAAATA AAATAAGTCA GCACATTCCC AGCCTATATC 4200
4201 TTCCTTGGAT TTAGCTTCTG CAAGTTCATC AGCTTCCTCC CTAATTTTAG CGTTCAACAA 4260
4261 AACTTCGTCC TCAAATAACC GTTTGGTATA AGAACCTTCT GGAGCATTGC TCTTACGATC 4320
4321 CCACAAGGTG GCTTCCATGG CTCTAAGACC CTTTGATTGG CCAAAACAGG AAGTGCGTTC 4380
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4561 TTTGGACGAG TACACCAGGC CCAACGAGTA ATTAGAAGAG TCAGCCACCA AAGTAGTGAA 4620
4621 TAGACCATCG GGGCGGTCAG TAGTCAAAGA CGCCAACAAA ATTTCACTGA CAGGGAACTT 4680
4681 TTTGACATCT TCAGAAAGTT CGTATTCAGT AGTCAATTGC CGAGCATCAA TAATGGGGAT 4740
4741 TATACCAGAA GCAACAGTGG AAGTCACATC TACCAACTTT GCGGTCTCAG AAAAAGCATA 4800
4801 AACAGTTCTA CTACCGCCAT TAGTGAAACT TTTCAAATCG CCCAGTGGAG AAGAAAAAGG 4860
4861 CACAGCGATA CTAGCATTAG CGGGCAAGGA TGCAACTTTA TCAACCAGGG TCCTATAGAT 4920
4921 AACCCTAGCG CCTGGGATCA TCCTTTGGAC AACTCTTTCT GCCAAATCTA GGTCCAAAAT 4980
4981 CACTTCATTG ATACCATTAT TGTACAACTT GAGCAAGTTG TCGATCAGCT CCTCAAATTG 5040
5041 GTCCTCTGTA ACGGATGACT CAACTTGCAC ATTAACTTGA AGCTCAGTCG ATTGAGTGAA 5100
5101 CTTGATCAGG TTGTGCAGCT GGTCAGCAGC ATAGGGAAAC ACGGCTTTTC CTACCAAACT 5160
5161 CAAGGAATTA TCAAACTCTG CAACACTTGC GTATGCAGGT AGCAAGGGAA ATGTCATACT 5220
5221 TGAAGTCGGA CAGTGAGTGT AGTCTTGAGA AATTCTGAAG CCGTATTTTT ATTATCAGTG 5280
5281 AGTCAGTCAT CAGGAGATCC TCTACGCCGG ACGCATCGTG GCCGGCATCA CCGGCGCCAC 5340
5341 AGGTGCGGTT GCTGGCGCCT ATATCGCCGA CATCACCGAT GGGGAAGATC GGGCTCGCCA 5400
5401 CTTCGGGCTC ATGAGCGCTT GTTTCGGCGT GGGTATGGTG GCAGGCCCCG TGGCCGGGGG 5460
5461 ACTGTTGGGC GCCATCTCCT TGCATGCACC ATTCCTTGCG GCGGCGGTGC TCAACGGCCT 5520
5521 CAACCTACTA CTGGGCTGCT TCCTAATGCA GGAGTCGCAT AAGGGAGAGC GTCGAGTATC 5580
5581 TATGATTGGA AGTATGGGAA TGGTGATACC CGCATTCTTC AGTGTCTTGA GGTCTCCTAT 5640
5641 CAGATTATGC CCAACTAAAG CAACCGGAGG AGGAGATTTC ATGGTAAATT TCTCTGACTT 5700
5701 TTGGTCATCA GTAGACTCGA ACTGTGAGAC TATCTCGGTT ATGACAGCAG AAATGTCCTT 5760
5761 CTTGGAGACA GTAAATGAAG TCCCACCAAT AAAGAAATCC TTGTTATCAG GAACAAACTT 5820
5821 CTTGTTTCGA ACTTTTTCGG TGCCTTGAAC TATAAAATGT AGAGTGGATA TGTCGGGTAG 5880
5881 GAATGGAGCG GGCAAATGCT TACCTTCTGG ACCTTCAAGA GGTATGTAGG GTTTGTAGAT 5940
5941 ACTGATGCCA ACTTCAGTGA CAACGTTGCT ATTTCGTTCA AACCATTCCG AATCCAGAGA 6000
6001 AATCAAAGTT GTTTGTCTAC TATTGATCCA AGCCAGTGCG GTCTTGAAAC TGACAATAGT 6060
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6121 TGACGAGCCA AGGCGATAAA TACCCAAATC TAAAACTCTT TTAAAACGTT AAAAGGACAA 6180
6181 GTATGTCTGC CTGTATTAAA CCCCAAATCA GCTCGTAGTC TGATCCTCAT CAACTTGAGG 6240
6241 GGCACTATCT TGTTTTAGAG AAATTTGCGG AGATGCGATA TCGAGAAAAA GGTACGCTGA 6300
6301 TTTTAAACGT GAAATTTATC TCAAGATCTC TGCCTCGCGC GTTTCGGTGA TGACGGTGAA 6360
6361 AACCTCTGAC ACATGCAGCT CCCGGAGACG GTCACAGCTT GTCTGTAAGC GGATGCCGGG 6420
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6601 ACCGCATCAG GCGCTCTTCC GCTTCCTCGC TCACTGACTC GCTGCGCTCG GTCGTTCGGC 6660
6661 TGCGGCGAGC GGTATCAGCT CACTCAAAGG CGGTAATACG GTTATCCACA GAATCAGGGG 6720
6721 ATAACGCAGG AAAGAACATG TGAGCAAAAG GCCAGCAAAA GGCCAGGAAC CGTAAAAAGG 6780
6781 CCGCGTTGCT GGCGTTTTTC CATAGGCTCC GCCCCCCTGA CGAGCATCAC AAAAATCGAC 6840
6841 GCTCAAGTCA GAGGTGGCGA AACCCGACAG GACTATAAAG ATACCAGGCG TTTCCCCCTG 6900
6901 GAAGCTCCCT CGTGCGCTCT CCTGTTCCGA CCCTGCCGCT TACCGGATAC CTGTCCGCCT 6960
6961 TTCTCCCTTC GGGAAGCGTG GCGCTTTCTC AATGCTCACG CTGTAGGTAT CTCAGTTCGG 7020
7021 TGTAGGTCGT TCGCTCCAAG CTGGGCTGTG TGCACGAACC CCCCGTTCAG CCCGACCGCT 7080
7081 GCGCCTTATC CGGTAACTAT CGTCTTGAGT CCAACCCGGT AAGACACGAC TTATCGCCAC 7140
7141 TGGCAGCAGC CACTGGTAAC AGGATTAGCA GAGCGAGGTA TGTAGGCGGT GCTACAGAGT 7200
7201 TCTTGAAGTG GTGGCCTAAC TACGGCTACA CTAGAAGGAC AGTATTTGGT ATCTGCGCTC 7260
7261 TGCTGAAGCC AGTTACCTTC GGAAAAAGAG TTGGTAGCTC TTGATCCGGC AAACAAACCA 7320
7321 CCGCTGGTAG CGGTGGTTTT TTTGTTTGCA AGCAGCAGAT TACGCGCAGA AAAAAAGGAT 7380
7381 CTCAAGAAGA TCCTTTGATC TTTTCTACGG GGTCTGACGC TCAGTGGAAC GAAAACTCAC 7440
7441 GTTAAGGGAT TTTGGTCATG AGATTATCAA AAAGGATCTT CACCTAGATC CTTTTACGCG 7500
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7561 CTTGCCAGCG CCCTAGCGCC CGCTCCTTTC GCTTTCTTCC CTTCCTTTCT CGCCACGTTC 7620
7621 GCCGGCTTTC CCCGTCAAGC TCTAAATCGG GGGCTCCCTT TAGGGTTCCG ATTTAGTGCT 7680
7681 TTACGGCACC TCGACCCCAA AAAACTTGAT TAGGGTGATG GTTCACGTAG TGGGCCATCG 7740
7741 CCCTGATAGA CGGTTTTTCG CCCTTTGACG TTGGAGTCCA CGTTCTTTAA TAGTGGACTC 7800
7801 TTGTTCCAAA CTGGAACAAC ACTCAACCCT ATCTCGGTCT ATTCTTTTGA TTTATAAGGG 7860
7861 ATTTTGCCGA TTTCGGCCTA TTGGTTAAAA AATGAGCTGA TTTAACAAAA ATTTAACGCG 7920
7921 AATTTTAACA AAATATTAAC GTTTACAATT TAAATCAATC TAAAGTATAT ATGAGTAAAC 7980
7981 TTGGTCTGAC AGTTACCAAT GCTTAATCAG TGAGGCACCT ATCTCAGCGA TCTGTCTATT 8040
8041 TCGTTCATCC ATAGTTGCCT GACTCCCCGT CGTGTAGATA ACTACGATAC GGGAGGGCTT 8100
8101 ACCATCTGGC CCCAGTGCTG CAATGATACC GCGAGACCCA CGCTCACCGG CTCCAGATTT 8160
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8221 CGCCTCCATC CAGTCTATTA ATTGTTGCCG GGAAGCTAGA GTAAGTAGTT CGCCAGTTAA 8280
8281 TAGTTTGCGC AACGTTGTTG CCATTGCTGC AGGCATCGTG TGTCACGCT CGTCGTTTGG 8340
8341 TATGGCTTCA TTCAGCTCCG GTTCCCAACG ATCAAGGCGA GTTACATGAT CCCCCATGTT 8400
8401 GTGCAAAAAA GCGGTTAGCT CCTTCGGTCC TCCGATCGTT GTCAGAAGTA AGTTGGCCGC 8460
8461 AGTGTTATCA CTCATGGTTA TGGCAGCACT GCATAATTCT CTTACTGTCA TGCCATCCGT 8520
8521 AAGATGCTTT TCTGTGACTG GTGAGTACTC AACCAAGTCA TTCTGAGAAT AGTGTATGCG 8580
8581 GCGACCGAGT TGCTCTTGCC CGGCGTCAAC ACGGGATAAT ACCGCGCCAC ATAGCAGAAC 8640
8641 TTTAAAAGTG CTCATCATTG GAAAACGTTC TTCGGGGCGA AAACTCTCAA GGATCTTACC 8700
8701 GCTGTTGAGA TCCAGTTCGA TGTAACCCAC TCGTGCACCC AACTGATCTT CAGCATCTTT 8760
8761 TACTTTCACC AGCGTTTCTG GGTGAGCAAA AACAGGAAGG CAAAATGCCG CAAAAAAGGG 8820
8821 AATAAGGGCG ACACGGAAAT GTTGAATACT CATACTCTTC CTTTTTCAAT ATTATTGAAG 8880
8881 CATTTATCAG GGTTATTGTC TCATGAGCGG ATACATATTT GAATGTATTT AGAAAAATAA 8940
8941 ACAAATAGGG GTTCCGCGCA CATTTCCCCG AAAAGTGCCA CCTGACGTCT AAGAAACCAT 9000
9001 TATTATCATG ACATTAACCT ATAAAAATAG GCGTATCACG AGGCCCTTTC GTCTTCAAGA 9060
9061 TTTCTCATGT TTGACAGCTT ATCATCGAAT TAATTCTCAT GTTTGACAGC TTATCATCGA 9120
9121 TAAGCTGACT CATGTTGGTA TTGTGAAATA GACGCAGATC GGGAACACTG AAAAATAACA 9180
9181 GTTATTATTC G                                                      9191
          |  10       |  20       |  30       |  40       |  50       |  60
```

Figure 18

DNA Strider 1.0 ### Wednesday, January 17, 1996 9:10:57 PM

~~pPIC9/NM3/261~~--> List (pnv 342)

DNA sequence 8974 b.p. AGATCTAACATC ... AGTTATTATTCG linear

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    121 TGCAAACGCA GGACCTCCAC TCCTCTTCTC CTCAACACCC ACTTTTGCCA TCGAAAAACC 180
    181 AGCCCAGTTA TTGGGCTTGA TTGGAGCTCG CTCATTCCAA TTCCTTCTAT TAGGCTACTA 240
    241 ACACCATGAC TTTATTAGCC TGTCTATCCT GGCCCCCCTG CCGAGGTTCA TGTTTGTTTA 300
    301 TTTCCGAATG CAACAAGCTC CGCATTACAC CCGAACATCA CTCCAGATGA GGGCTTTCTG 360
    361 AGTGTGGGGT CAAATAGTTT CATGTTCCCc AAATGGCCCA AAACTGACAG TTTAAACCCT 420
    421 GTCTTGGAAC CTAATATGAC AAAAGCGTGA TCTCATCCAA GATGAACTAA GTTTGGTTCG 480
    481 TTGAAATGCT AACGGCCAGT TGGTCAAAAA GAAACTTCCA AAAGTCGCCA TACCGTTTGT 540
    541 CTTGTTTGGT ATTGATTGAC GAATGCTCAA AAATAATCTC ATTAATGCTT AGCGCAGTCT 600
    601 CTCTATCGCT TCTGAACCCG GTGGCACCTG TGCCGAAACG CAAATGGGGA AACaCCCGCT 660
    661 TTTTGGATGA TTATGCATTG TcTCCACATT GTATGCTTCC AAGATTCTGG TGGGAATACT 720
    721 GCTGATAGCC TAACGTTCAT GATCAAAATT TAACTGTTCT AACCCCTACT TGACAGcAAT 780
    781 ATATAAACAG AAGGAAGCTG CCCTGTCTTA AACCTTTTTT TTTATCATCA TTATTAGCTT 840
    841 ACTTTCATAA TTGCGACTGG TTCCAATTGA CAAGCTTTTG ATTTTAACGA CTTTTAACGA 900
    901 CAACTTGAGA AGATCAAAAA ACAACTAATT ATTCGAAGGA TCCAAACGat gAGATTTCCT 960
    961 TCAATTTTTA CTGCAGTTTT ATTCGCAGCA TCCTCCGCAT TAGCTGCTCC AGTCAACACT 1020
   1021 ACAACAGAAG ATGAAACGGC ACAAATTCCG GCTGAAGCTG TCATCGGTTA CTCAGATTTA 1080
   1081 GAAGGGGATT TCGATGTTGC TGTTTTGCCA TTTTCCAACA GCACAAATAA CGGGTTATTG 1140
   1141 TTTATAAATA CTACTATTGC CAGCATTGCT GCTAAAGAAG AAGGGGTATC TCTCGAGAAA 1200
   1201 AGAGAGGCTG AAGCTTACGT AGAATTCgac gtcactttgt acggtactat taaggctggt 1260
   1261 gttgagactt cccgctctgt atttcaccag aacggccaag ttactgaagt tacaaccgct 1320
   1321 accggcatcg ttgatttggg ttcgaaaatc ggcttcaaag gccaagaaga cctcggtaac 1380
   1381 ggcctgaaag ccatttggca ggttgagcaa aaagcatcta tcgccggtac tgactccggt 1440
   1441 tggggcaacc gccaatcctt catcggcttg aaaggcggct tcggtaaatt gcgcgtcggt 1500
   1501 cgtttgaaca gcgtcctgaa agacaccggc gacatcaatc cttgggatag caaaagcgac 1560
   1561 tatttgggtg taaacaaaat tgccgaaccc gaggcacgcc tcatttccgt acgctacgat 1620
   1621 tctcccgaat ttgccggcct cagcggcagc gtacaatacg cgcttaacga caatgcaggc 1680
   1681 agacataaca gcgaatctta ccacgccggc ttcaactaca aaaacggtgg cttcttcgtg 1740
   1741 caatatggcg gtgcctataa aagacatcat caagtgcaag agggcttgaa tattgagaaa 1800
   1801 taccagattc accgtttggt cagcggttac gacaatgatg ccctgtacgc ttccgtagcc 1860
   1861 gtacagcaac aagacgcgaa actgactgat gcttccaatt cgcacaactc tcaaaccgaa 1920
   1921 gttgccgcta ccttggcata ccgcttcggc aacgtaacgc cccgagtttc ttacgcccac 1980
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   2041 ggtgcggaat acgacttctc caaacgcact tctgccttgg tttctgccgg ttggttgcaa 2100
   2101 gaaggcaaag gcgaaaacaa attcgtagcg actgccggcg gtgttggtct gcgtcacaaa 2160
   2161 ttctaaCCTA GGGCGGCCGC GAATTAATTC GCCTTAGACA TGACTGTTCC TCAGTTCAAG 2220
   2221 TTGGGCAcTT ACGAGAAGAC CGGTCTTGCT AGATTCTAAT CAAGAGGATG TCAGAATGCC 2280
   2281 ATTTGCCTGA GAGATGCAGG CTTCATTTTT GATACTTTTT TATTTGTAAC CTATATAGTA 2340
   2341 TAGGATTTTT TTTGTCATTT TGTTTCTTCT CGTACGAGCT TGCTCCTGAT CAGCCTATCT 2400
   2401 CGCAGCTGAT GAATATCTTG TGGTAGGGGT TTGGGAAAAT CATTCGAGTT TGATGTTTTT 2460
   2461 CTTGGTATTT CCCACTCCTC TTCAGAGTAC AGAAGATTAA GTGAGAAGTT CGTTTGTGCA 2520
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   2581 CACCGTGTAT GAAATCTAAC AATGCGCTCA TCGTCATCCT CGGCACCGTC ACCCTGGATG 2640
   2641 CTGTAGGCAT AGGCTTGGTT ATGCCGGTAC TGCCGGGCCT CTTGCGGGAT ATCGTCCATT 2700
   2701 CCGACAGCAT CGCCAGTCAC TATGGCGTGC TGCTAGCGCT ATATGCGTTG ATGCAATTTC 2760
   2761 TATGCGCACC CGTTCTCGGA GCACTGTCCG ACCGCTTTGG CCGCCGCCCA GTCCTGCTCG 2820
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   3001 ACTGCTTGGC CAATATGTTT CAGTCCCTCA GGAGTTACGT CTTGTGAAGT GATGAACTTC 3060
   3061 TGGAAGGTTG CAGTGTTAAC TCCGCTGTAT TGACGGGCAT ATCCGTACGT TGGCAAAGTG 3120
   3121 TGGTTGGTAC CGGAGGAGTA ATCTCCACAA CTCTCTGGAG AGTAGGCACC AACAAACACA 3180
   3181 GATCCAGCGT GTTGTACTTG ATCAACATAA GAAGAAGCAT TCTCGATTTG CAGGATCAAG 3240
   3241 TGTTCAGGAG CGTACTGATT GGACATTTCC AAAGCCTGCT CGTAGGTTGC AACCGATAGG 3300
   3301 GTTGTAGAGT GTGCAATACA CTTGCGTACA ATTTCAACCC TTGGCAACTG CACAGCTTGG 3360
   3361 TTGTGAACAG CATCTTCAAT TCTGGCAAGC TCCTTGTCTG TCATATCGAC AGCCAACAGA 3420
   3421 ATCACCTGGG AATCAATACC ATGTTCAGCT TGAGACAGAA GGTCTGAGGC AACGAAATCT 3480
   3481 GGATCAGCGT ATTTATCAGC AATAACTAGA ACTTCAGAAG GCCCAGCAGC CATGTCAATA 3540
   3541 CTACACAGGC CTGATGTGTC ATTTTGAACC ATCATCTTGG CAGCAGTAAC GAACTGGTTT 3600
   3601 CCTGGACCAA ATATTTTGTC ACACTTAGGA ACAGTTTCTG TTCCGTAAGC CATAGCAGCT 3660
   3661 ACTGCCTGGC CGCCTCCTGC TAGCACGATA CACTTAGCAC CAACCTTGTG GGCAACGTAG 3720
   3721 ATGACTTCTG GGCTAAGGGT ACCATCCTTC TTAGGTGGAG ATGCAAAAAC AATTTCTTTG 3780
   3781 CAACCAGCAA CTTTGGCAGG AACACCCAGC ATCAGGGAAG TGGAAGGCAG AATTGCGGTT 3840
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Fig. 18I

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3901 CCAGGGCAAG TCTCAACTTG CAACGTCTCC GTTAGTTGAG CTTCATGGAA TTTCCTGACG 3960
3961 TTATCTATAG AGAGATCAAT GGCTCTCTTA ACGTTATCTG GCAATTGCAT AAGTTCCTCT 4020
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5761 AACGGCCTCA ACCTACTACT GGGCTGCTTC CTAATGCAGG AGTCGCATAA GGGAGAGCGT 5820
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7741 TTTaaAttaa aaatgaagtt ttaaatcaat ctaaagtata tatgagtaaa cttggtctga 7800
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7861 catagttgcc tgactccccg tcgtgtagat aactacgata cgggagggct taccatctgg 7920
7921 ccccagtgct gcaatgatac cgcgagaccc acgctcaccg gctccagatt tatcagcaat 7980
7981 aaaccagcca gccggaaggg ccgagcgcag aagtggtcct gcaactttat ccgcctccat 8040
8041 ccagtctatt aattgttgcc gggaagctag agtaagtagt tcgccagtta atagtttgcg 8100
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FIG 18C

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8401 ttgctcttgc ccggcgtcaa cacgggataa taccgcgcca catagcagaa ctttaaaagt 8460
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8581 cagcgtttct gggtgagcaa aaacaggaag gcaaaatgcc gcaaaaaagg gaataagggc 8640
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8761 ggttccgcgc acatttcccc gaaaagtgcc acctgacgtc taagaaacca ttattatcat 8820
8821 gacattaacc tataaaaata ggcgtatcac gaggcccttt cgtcttcaaG AATTAATTCT 8880
8881 CATGTTTGAC AGCTTATCAT CGATAAGCTG ACTCATGTTG GTATTGTGAA ATAGACGCAG 8940
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Figure 19f

DNA Strider 1.0 ### Wednesday, January 17, 1996 9:30:46 PM

>PIC-9K/MB3/61 -> List (pnv 350)

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 121 TGCAAACGCA GGACCTCCAC TCCTCTTCTC CTCAACACCC ACTTTTGCCA TCGAAAAACC 180
 181 AGCCCAGTTA TTGGGCTTGA TTGGAGCTCG CTCATTCCAA TTCCTTCTAT TAGGCTACTA 240
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1081 GAAGGGGATT TCGATGTTGC TGTTTTGCCA TTTTCCAACA GCACAAATAA CGGGTTATTG 1140
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3361 TTGTGAACAG CATCTTCAAT TCTGGCAAGC TCCTTGTCTG TCATATCGAC AGCCAACAGA 3420
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Fig. 1

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3961 TTATCTATAG AGAGATCAAT GGCTCTCTTA ACGTTATCTG GCAATTGCAT AAGTTCCTCT 4020
4021 GGGAAAGGAG CTTCTAACAC AGGTGTCTTC AAACGCACTC CATCAAACTT GGCAGTTAGT 4080
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4141 ATAATCTGTT CCGTTTTCTG GATAGGACGA CGAAGGGCAT CTTCAATTTC TTGTGAGGAG 4200
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4381 TTGGCACATC TAACTAATGC AAAATAAAAT AAGTCAGCAC ATTCCCAGGC TATATCTTCC 4440
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4681 AATTCGATAC CCAGCAACTT TTGAGTTGCT CCAGATGTAG CACCTTTATA CCACAAACCG 4740
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4801 GACGAGTACA CCAGGCCCAA CGAGTAATTA GAAGAGTCAG CCACCAAAGT AGTGAATAGA 4860
4861 CCATCGGGGC GGTCAGTAGT CAAAGACGCC AACAAAATTT CACTGACAGG GAACTTTTTG 4920
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5461 GTCGGACAGT GAGTGTAGTC TTGAGAAATT CTGAAGCCGT ATTTTTATTA TCAGTGAGTC 5520
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5581 CGCTGAGGTC TGCCTCGTGA AGAAGGTGTT GCTGACTCAT ACCAGGCCTG AATCGCCCCA 5640
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FIG 19

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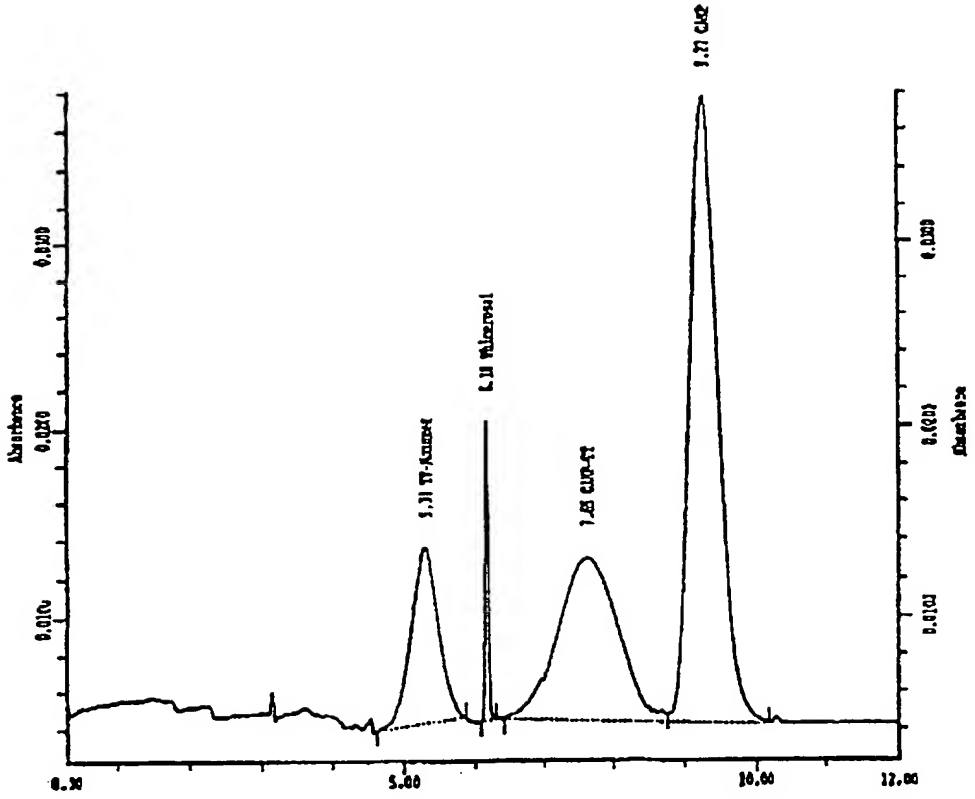

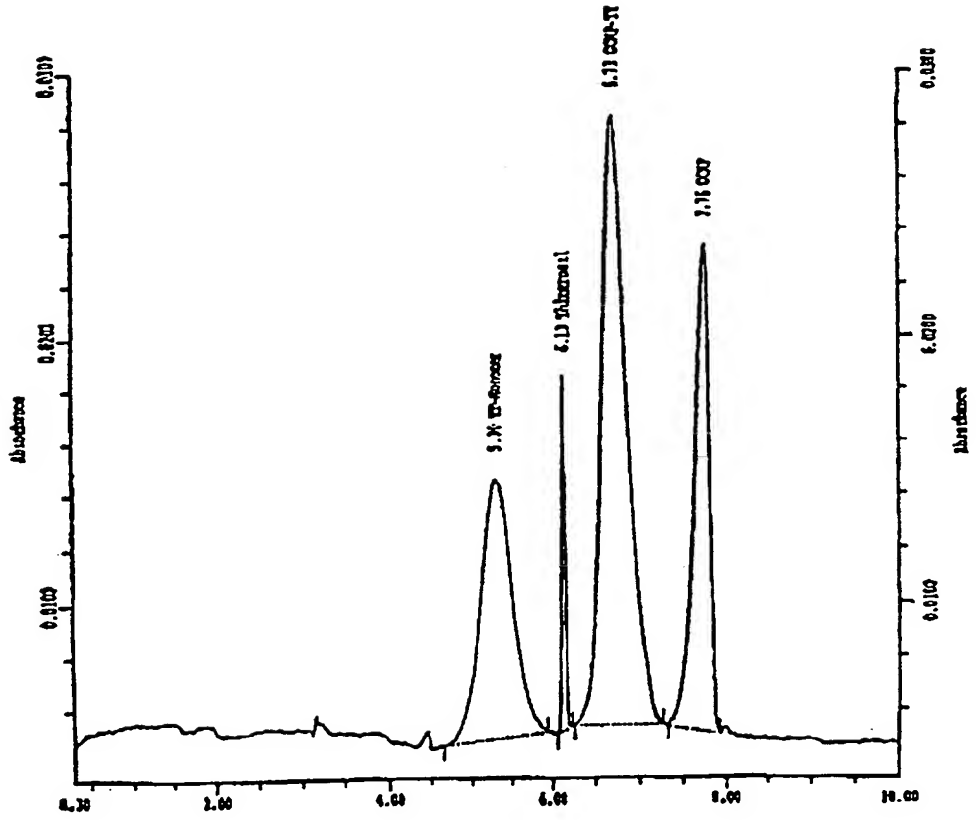

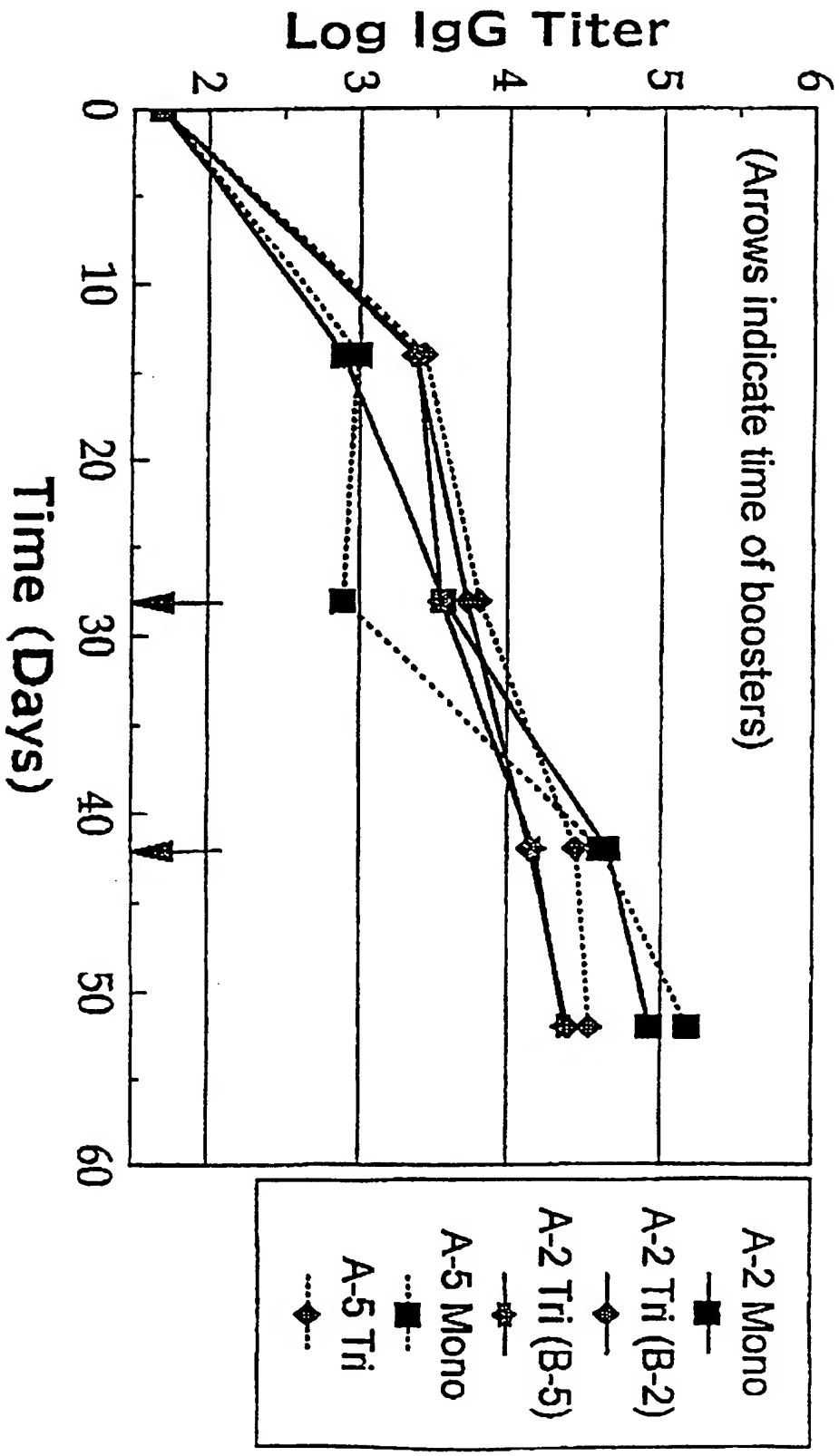

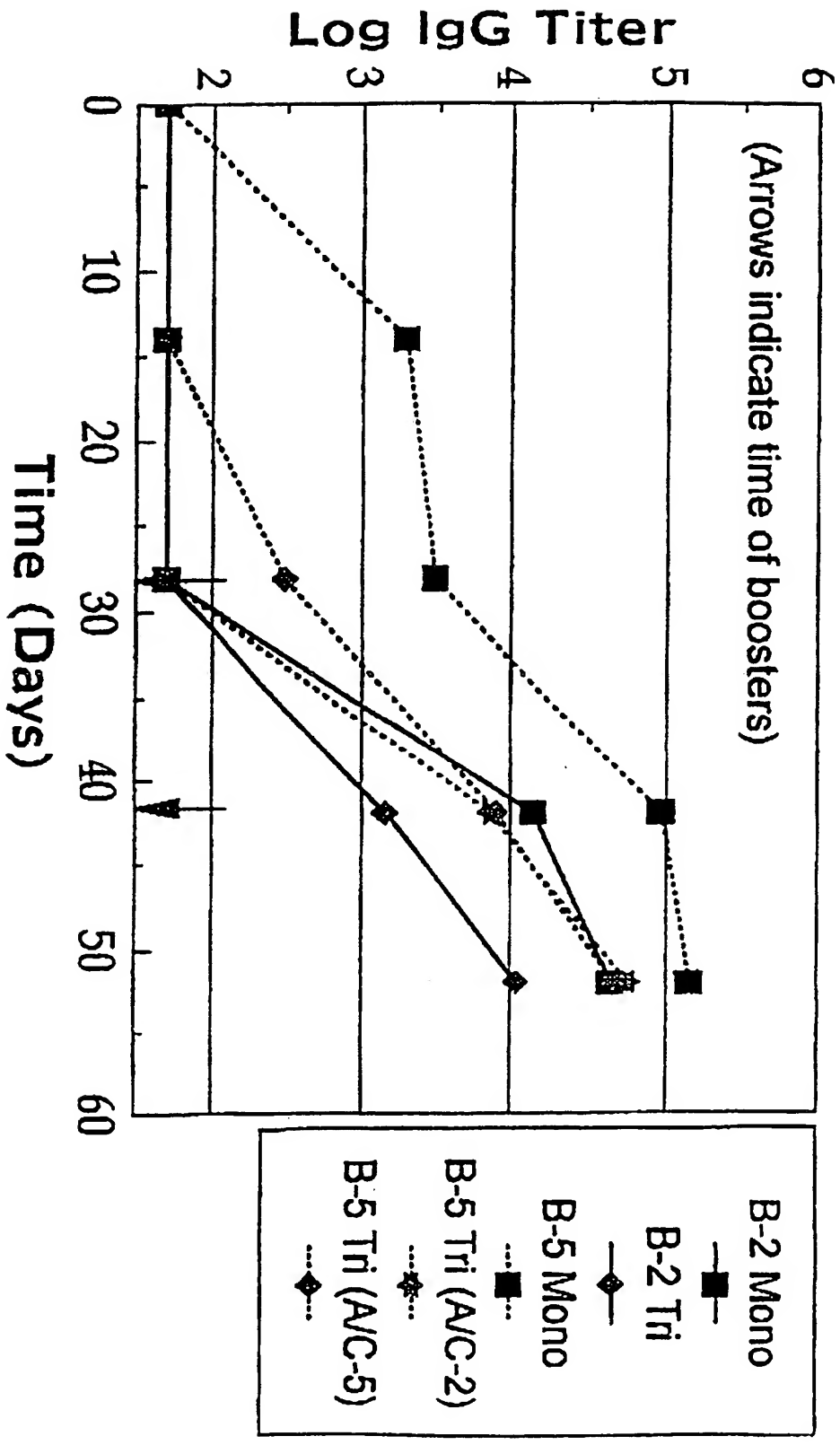
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(Arrows indicate time of boosters)
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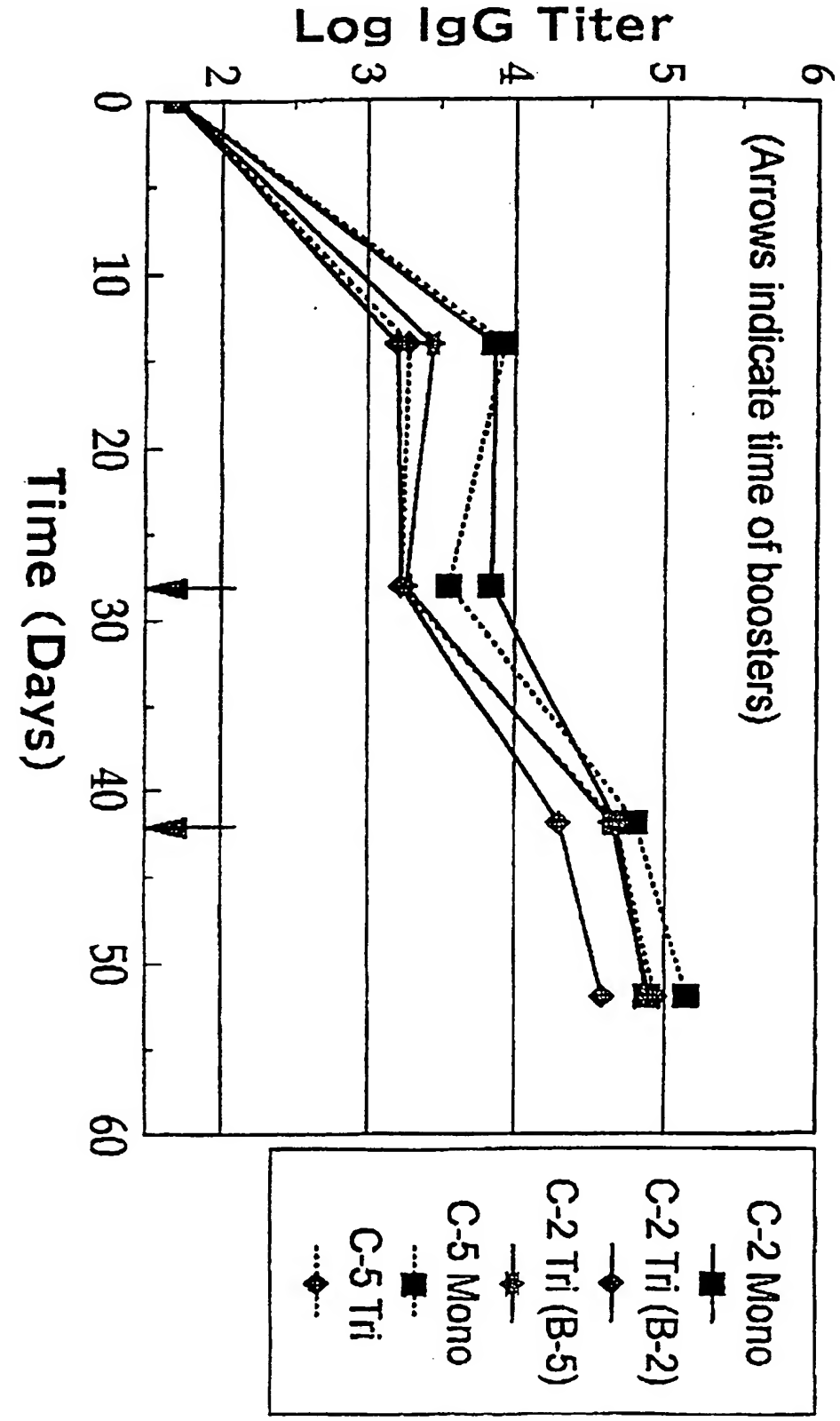

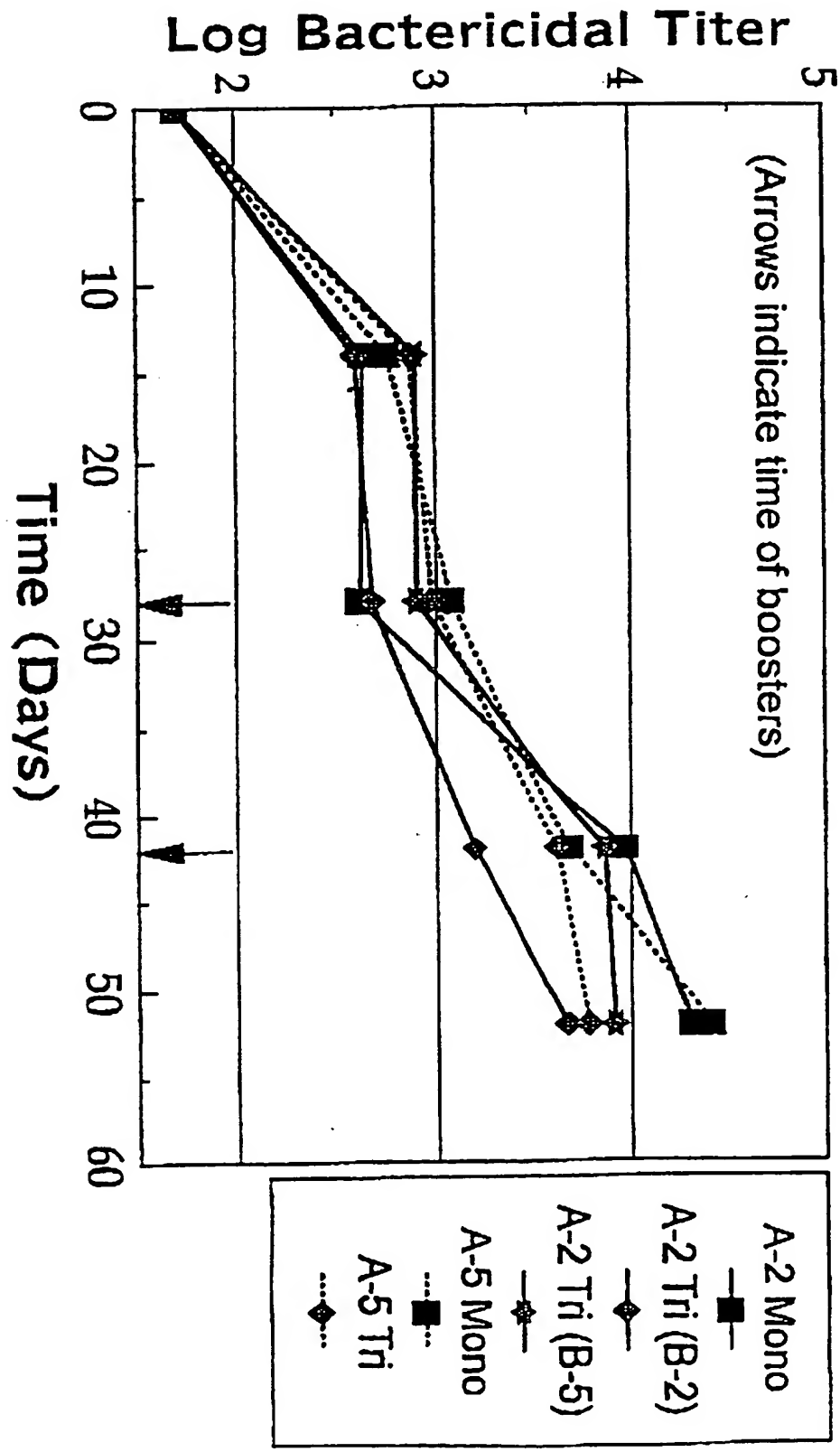
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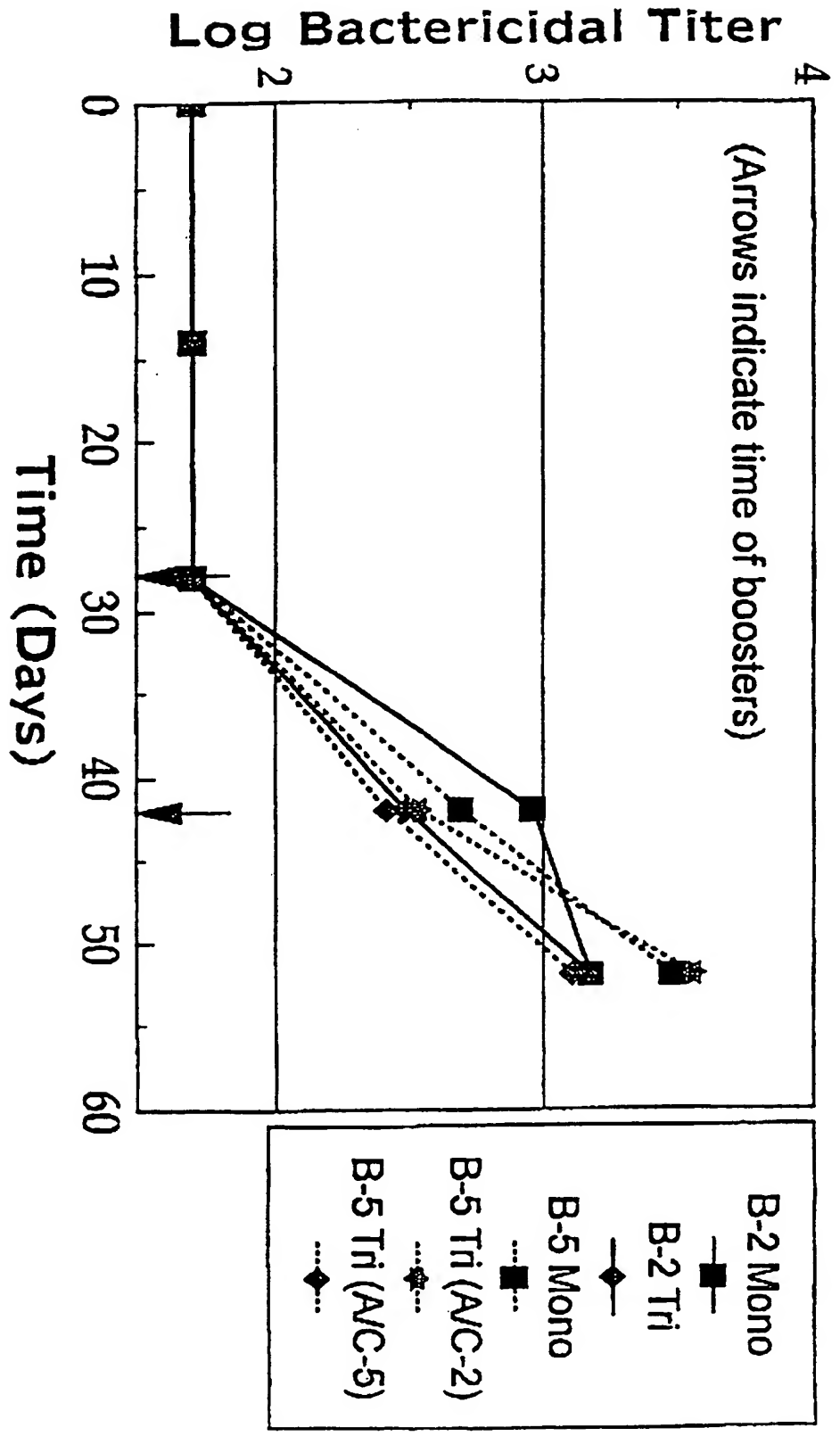

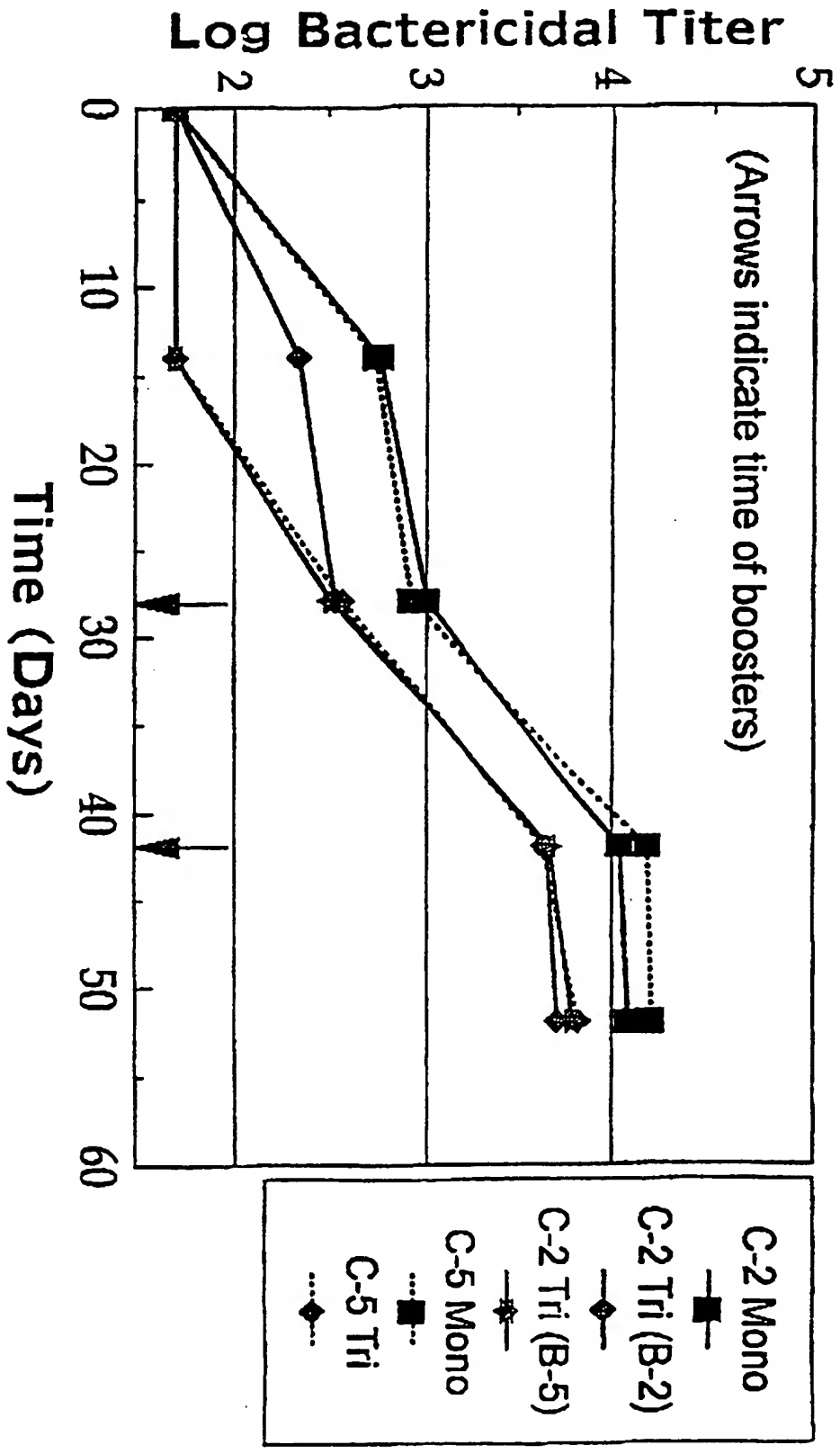

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/01687

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 69.7, 69.8, 69.9, 255.1, 320.1; 530/412, 416, 417; 536/23.7; 424/185.1, 192.1

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APS, MEDLINE, BIOSIS, CA, EMBASE, WPIDS
terms: meningococcal, porin, expression, group A, B, and C, pastoris, wobble

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 4,356,170 A (JENNINGS et al.) 26 October 1982 (26/10/82), see entire document.	23, 24, 26, 28-32 --- 25, 27, 33
Y	WO 95/03413 A1 (THE ROCKEFELLER UNIVERSITY) 02 February 1995 (02.02.95), see entire document.	1-33
Y	US 5,268,273 A (BUCKHOLZ) 07 December 1993 (07/12/93), see entire document.	1-22

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/01687

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BLACHLY-DYSON et al. Cloning and Functional Expression in Yeast of Two Human Isoforms of the Outer Mitochondrial Membrane Channel, the Voltage-dependent Anion Channel. Journal of Biological Chemistry. 25 January 1993 Vol. 268, No. 3, pages 1835-1841.	1-22
Y	CHOI et al. Study of Putative Glycosylation Sites in Bovine β-Casein Introduced by PCR-Based Site-Directed Mutagenesis. J. Agric. Food Chem.. January 1996, Vol. 44, No. 1, pages 358-364.	1-22
Y	BENNETZEN et al. Codon Selection in Yeast. Journal of Biological Chemistry. 25 March 1982, Vol. 257, No. 6, pages 3026-3031.	1-22
Y	MITRA. YEAST tRNA (ANTICODON CUU) TRANSLATES AAA CODON. FEBS Letters. July 1978, Volumn 91, Number 1, pages 78-80, see entire document.	1-22
Y	HALSTENSEN et al. Human Opsonins to Meningococci After Vaccination. Infection and Immunity. December 1984, Vol. 46, No. 3, pages 673-676, see entire document.	23-33
Y	WO 92/04915 A1 (NORTH AMERICAN VACCINE, INC.) 02 April 1992 (02.04.92), see entire document.	23-33
Y	JENNINGS et al. Induction of Meningococcal Group B Polysaccharide-Specific IgG Antibodies In Mice By Using An N-Propionylated B Polysaccharide-Tetanus Conjugate Vaccine. Journal of Immunology. 01 September 1986, Vol. 137, No. 5, pages 1708-1713, see entire document.	25, 27

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(54) Title: EXPRESSION OF GROUP B NEISSERIA MENINGITIDIS OUTER MEMBRANE (MB3) PROTEIN FROM YEAST AND VACCINES

(57) Abstract

The present invention relates, in general, to a method for obtaining the outer membrane protein meningococcal group B porin proteins, in particular MB3, and fusion proteins thereof. In particular, the present invention relates to a method of expressing the outer membrane protein meningococcal group B porin proteins in yeast. The invention also relates to a method of high level expression of the above-mentioned proteins wherein the rate of protein expression is enhanced by substituting a nucleotide sequence for the 5' region of the gene encoding said protein wherein the sequence has been optimized for yeast codon usage. The invention also relates to a vaccine comprising group A meningococcal polysaccharide (GAMP), group B meningococcal polysaccharide (GBMP) and group C meningococcal polysaccharide (GCMP) antigens, together with a pharmaceutically acceptable carrier. The invention also relates to a method of inducing an immune response in a mammal, comprising administering the above-mentioned vaccine to a mammal in an amount sufficient to induce an immune response.

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Expression of Group B *Neisseria meningitidis* Outer Membrane (MB3) Protein from Yeast and Vaccines

Background of the Invention

Field of the Invention

The present invention is in the field of recombinant genetics, protein expression, and vaccines. The present invention relates to a method of expressing in a recombinant yeast host an outer membrane group B porin protein from *Neisseria meningitidis*. The invention also relates to a vaccine comprising group A meningococcal polysaccharide (GAMP), group B meningococcal polysaccharide (GBMP) and group C meningococcal polysaccharide (GCMP) antigens, together with a pharmaceutically acceptable carrier. The invention also relates to a method of inducing an immune response in a mammal, comprising administering the above-mentioned vaccine to a mammal in an amount sufficient to induce an immune response.

Background Information

Meningococcal meningitis remains a worldwide problem, and occurs in both endemic and epidemic forms (Peltola, H., *Rev. Infect. Dis.* 5:71-91 (1983); Gotschlich, E.C., "Meningococcal Meningitis," in *Bacterial Vaccines*, Germanier, E., ed., Academic, New York (1984), pp.237-255). Epidemic disease occurs in

all parts of the world and incidences as high as 500 per 100,000 population have been reported. Without antibiotic treatment the mortality is extremely high (85%), and even with antibiotics, it remains at approximately 10%. In addition, patients cured by antibiotic therapy can still suffer serious and permanent neurologic deficiencies. These facts together with the emergence of sulfadiazine-resistant strains of *Neisseria meningitidis* promoted the rapid development of a commercial vaccine (Peltola, H., *Rev. Infect. Dis.* 5:71-91 (1983)).

Neisseria meningitidis is a gram-negative organism that has been classified serologically into groups A, B, 29e, W135, X, Y, and Z (Gotschlich, E.C., "Meningococcal Meningitis," in *Bacterial Vaccines*, Germanier, E., ed., Academic, New York (1984), pp.237-255). Additional groups H, I, and K were isolated in China (Ding, S.-Q. *et al.*, *J. Biol. Stand.* 9:307-315 (1981)) and group L was isolated in Canada (Ashton, F.E. *et al.*, *J. Clin. Microbiol.* 17:722-727 (1983)). The grouping system is based on the organisms' capsular polysaccharides. It was established (Lui, T.-Y. *et al.*, *J. Biol. Chem.* 246:2849-2858 (1971)) that the group A polysaccharide is a partially O-acetylated (1→6) linked homopolymer of 2-acetamido-2-detoxy-D-mannopyranosyl phosphate, and that both groups B and C polysaccharides are homopolymers of sialic acid.

N. meningitidis groups A, B, and C are responsible for approximately 90% of cases of meningococcal meningitis. Success in the prevention of group A and C meningococcal meningitis was achieved using a bivalent polysaccharide vaccine (Gotschlich, E.C. *et al.*, *J. Exp. Med.* 129:1367-1384 (1969); Artenstein, M.S. *et al.*, *N. Engl. J. Med.* 282:417-420 (1970)); this vaccine became a commercial product and has been used successfully in the last decade in the prevention and arrest of major meningitis epidemics in many parts of the world. However, there has been a need to augment this vaccine because a significant proportion of cases of meningococcal meningitis are due to groups other than A and C. Group B is of particular epidemiologic importance, but groups Y and W135 are also significant (Cadoz, M. *et al.*, *Vaccine* 3:340-342 (1985)). The inclusion of the group B polysaccharide in the vaccine has been a special problem

(see below); however, a tetravalent vaccine comprising groups A, C, W135, and Y has proven to be safe and immunogenic in humans (Cadoz, M. *et al.*, *Vaccine 3*:340-342 (1985)) and is the currently used meningococcal meningitis vaccine (Jennings, H.J., "Capsular Polysaccharides as Vaccine Candidates," in *Current Topics in Microbiol. and Immunol.*, Jann, D. and Jann, B., eds, Springer-Verlag, Berlin (1990) Vol 150:97-127).

The outer membranes of *Neisseria* species much like other Gram negative bacteria are semi-permeable membranes which allow free flow access and escape of small molecular weight substances to and from the periplasmic space of these bacteria but retard molecules of larger size (Heasley, F.A., *et al.*, "Reconstitution and characterization of the *N. gonorrhoeae* outer membrane permeability barrier," in *Genetics and Immunobiology of Neisseria gonorrhoeae*, Danielsson and Normark, eds., University of Umea, Umea, pp. 12-15 (1980); Douglas, J.T., *et al.*, *FEMS Microbiol. Lett. 12*:305-309 (1981)). One of the mechanisms whereby this is accomplished is the inclusion within these membranes of proteins which have been collectively named porins. These proteins are made up of three identical polypeptide chains (Jones, R.B., *et al.*, *Infect. Immun. 30*:773-780 (1980); McDade, Jr. and Johnston, *J. Bacteriol. 141*:1183-1191 (1980)) and in their native trimer conformation, form water filled, voltage-dependent channels within the outer membrane of the bacteria or other membranes to which they have been introduced (Lynch, E.C., *et al.*, *Biophys. J. 41*:62 (1983); Lynch, E.C., *et al.*, *Biophys. J. 45*:104-107 (1984); Young, J.D.E., *et al.*, *Proc. Natl. Acad. Sci. USA 80*:3831-3835 (1983); Mauro, A., *et al.*, *Proc. Natl. Acad. Sci. USA 85*:1071-1075 (1988); Young, J.D., *et al.*, *Proc. Natl. Acad. Sci. USA 83*:150-154 (1986)). Because of the relative abundance of these proteins within the outer membrane, these protein antigens have also been used to subgroup both *Neisseria gonorrhoeae* and *Neisseria meningitidis* into several serotypes for epidemiological purposes (Frasch, C.E., *et al.*, *Rev. Infect. Dis. 7*:504-510 (1985); Knapp, J.S., *et al.*, "Overview of epidemiological and clinical applications of auxotype/serovar classification of *Neisseria gonorrhoeae*," *The Pathogenic*

Neisseriae, Schoolnik, G.K., ed., American Society for Microbiology, Washington, pp. 6-12 (1985)). To date, many of these proteins from both gonococci and meningococci have been purified (Heckels, J.E., *J. Gen. Microbiol. 99*:333-341 (1977); James and Heckels, *J. Immunol. Meth. 42*:223-228 (1981); Judd, R.C., *Anal. Biochem. 173*:307-316 (1988); Blake and Gotschlich, *Infect. Immun. 36*:277-283 (1982); Wetzler, L.M., *et al.*, *J. Exp. Med. 168*:1883-1897 (1988)), and cloned and sequenced (Gotschlich, E.C., *et al.*, *Proc. Natl. Acad. Sci. USA 84*:8135-8139 (1987); McGuinness, B., *et al.*, *J. Exp. Med. 171*:1871-1882 (1990); Carbonetti and Sparling, *Proc. Natl. Acad. Sci. USA 84*:9084-9088 (1987); Feavers, I.M., *et al.*, *Infect. Immun. 60*:3620-3629 (1992); Murakami, K., *et al.*, *Infect. Immun. 57*:2318-2323 (1989); Wolff and Stern, *FEMS Microbiol. Lett. 83*:179-186 (1991); Ward, M.J., *et al.*, *FEMS Microbiol. Lett. 73*:283-289 (1992)).

The porin proteins were initially co-isolated with lipopolysaccharides (LPS). Consequently, the porin proteins have been termed "endotoxin-associated proteins" (Bjornson *et al.*, *Infect. Immun. 56*:1602-1607 (1988)). Studies on the wild type porins have reported that full assembly and oligomerization are not achieved unless LPS from the corresponding bacterial strain is present in the protein environment (Holzenburg *et al.*, *Biochemistry 28*:4187-4193 (1989); Sen and Nikaido, *J. Biol. Chem. 266*:11295-11300 (1991)).

The meningococcal porins have been subdivided into three major classifications which in antedated nomenclature were known as Class 1, 2, and 3 (Frasch, C.E., *et al.*, *Rev. Infect. Dis. 7*:504-510 (1985)). Each meningococcus examined has contained one of the alleles for either a Class 2 porin gene or a Class 3 porin gene but not both (Feavers, I.M., *et al.*, *Infect. Immun. 60*:3620-3629 (1992)); Murakami, K., *et al.*, *Infect. Immun. 57*:2318-2323 (1989)). The presence or absence of the Class 1 gene appears to be optional. Likewise, all probed gonococci contain only one porin gene with similarities to either the Class 2 or Class 3 allele (Gotschlich, E.C., *et al.*, *Proc. Natl. Acad. Sci. USA 84*:8135-8139 (1987); Carbonetti and Sparling, *Proc. Natl. Acad. Sci. USA 84*:9084-9088

(1987)). *N. gonorrhoeae* appear to completely lack the Class 1 allele. The data from the genes that have been thus far sequenced would suggest that all neisserial porin proteins have at least 70% homology with each other with some variations on a basic theme (Feavers, I.M., *et al.*, *Infect. Immun. 60*:3620-3629 (1992)). It has been suggested that much of the variation seen between these neisserial porin proteins is due to the immunological pressures brought about by the invasion of these pathogenic organisms into their natural host, man. However, very little is known about how the changes in the porin protein sequence effect the functional activity of these proteins.

It has been previously reported that isolated gonococcal porins of the Class 2 allelic type behave electrophysically somewhat differently than isolated gonococcal porins of the Class 3 type in lipid bilayer studies both in regards to their ion selectivity and voltage-dependence (Lynch, E.C., *et al.*, *Biophys. J. 41*:62 (1983); Lynch, E.C., *et al.*, *Biophys. J. 45*:104-107 (1984)). Furthermore, the ability of the different porins to enter these lipid bilayers from intact living bacteria seems to correlate not only with the porin type but also with the neisserial species from which they were donated (Lynch, E.C., *et al.*, *Biophys. J. 45*:104-107 (1984)). It would seem that at least some of these functional attributes could be related to different areas within the protein sequence of the porin. One such functional area, previously identified within all gonococcal Class 2-like proteins, is the site of chymotrypsin cleavage. Upon chymotrypsin digestion, this class of porins lack the ability to respond to a voltage potential and close. Gonococcal Class 3-like porins as well as meningococcal porins lack this sequence and are thus not subject to chymotrypsin cleavage but nonetheless respond by closing to an applied voltage potential (Greco, F., "The formation of channels in lipid bilayers by gonococcal major outer membrane protein," thesis, The Rockefeller University, New York (1981); Greco, F., *et al.*, *Fed. Proc. 39*:1813 (1980)).

As the *Neisseria* porins are among the most abundant proteins present in the outer membrane of these organisms, and as they do not undergo antigenic

shift during infection (unlike several other major surface antigens), their universal presence in both *Neisseria meningitidis* and *Neisseria gonorrhoea*, as well as their exposure at the surface, make them candidates for components of vaccines against these organisms. Patients convalescing from meningococcal disease produce anti-porin antibodies, and antibodies elicited by immunization with porin proteins are bactericidal to homologous serotypes. Furthermore, within a particular epidemiologic setting, most strains causing meningococcal disease belong to a limited number of serotypes, notably serotype 2 among strains with a class 2 protein and serotype 15 among strains with class 3 proteins. Therefore, class 2 and 3 proteins are attractive candidates for vaccines.

The major impediment for such studies has been the ability to easily manipulate the porin genes by modern molecular techniques and obtain sufficient purified protein to carry out the biophysical characterizations of these altered porin proteins. It was early recognized that cloned neisserial porin genes, when expressed in *Escherichia coli*, were lethal to the host *E. coli* (Carbonetti and Sparling, *Proc. Natl. Acad. Sci. USA 84*:9084-9088 (1987); Carbonetti, N.H., *et al.*, *Proc. Natl. Acad. Sci. USA 85*:6841-6845 (1988); Barlow, A.K., *et al.*, *Infect. Immun. 55*:2734-2740 (1987)). Thus, many of these genes were cloned and sequenced as pieces of the whole gene or placed into low copy number plasmids under tight expression control (Carbonetti, N.H., *et al.*, *Proc. Natl. Acad. Sci. USA 85*:6841-6845 (1988)). Under these conditions, even when the entire porin gene was expressed, very little protein accumulated that could be further purified and processed for characterization.

Another tack to this problem which has met with a modicum of success has been to clone the porin genes into a low copy, tightly controlled expression plasmid, introduce modifications to the porin gene, and then reintroduce the modified sequence back into *Neisseria* (Carbonetti, N.H., *et al.*, *Proc. Natl. Acad. Sci. USA 85*:6841-6845 (1988)). However, this has also been fraught with problems due to the elaborate restriction endonuclease system present in

Neisseria, especially gonococci (Davies, J.K., *Clin. Microbiol. Rev. 2*:S78-S82 (1989)).

While a vaccine comprising neisserial porin has long been sought, an effective meningococcal polysaccharide vaccine which would give complete coverage to all serogroup organisms and to all humans is also needed. Several serious problems remain in the development of such a broad range polysaccharide vaccine. First, it has been established that although the group A and C polysaccharides are efficacious in adults and older children, their effectiveness in infants has only been marginal (Goldschneider, I., *et al., J. Infect. Dis. 128*:769-776 (1973); Gotschlich, E.C., *et al.*, "The Immune Responses to Bacterial Polysaccharides in Man." In: *Antibodies in Human Diagnosis and Therapy*, Haber, E. and Krause, R.M., eds., Raven, New York (1977), pp. 391-402). Second, the group B meningococcal polysaccharide is only poorly immunogenic in man (Wyle, F.A., *et al., J. Infect. Dis. 126*:514-521 (1972)). A third problem is the tendency for multivalent vaccines to be less immunogenic than each component would be if administered individually (Insel, R.A., "Potential alterations in immunogenicity by combining or simultaneously administering vaccine components." In: *Annals of the New York Academy of Sciences, Vol. 754. Combined Vaccines and Simultaneous Administration: Current Issues and Perspectives*, Williams, J.C., *et al.*, eds, New York Academy of Sciences, New York (1993), pp. 35-47; Clemens, J., *et al.*, "Interactions between PRP-T vaccine against *Haemophilus influenzae* type b and conventional infant vaccines: lessons for future studies of simultaneous immunization and combined vaccines," In: *Annals of the New York Academy of Sciences, Vol. 754. Combined Vaccines and Simultaneous Administration: Current Issues and Perspectives*, Williams, J.C., *et al.*, eds, New York Academy of Sciences, New York (1993), pp. 255-266; Paradiso, P.R., *et al., Pediatrics 92(6)*:827-832 (1993)).

Presently available vaccines against group A and C *N. meningitidis* are poorly immunogenic in human infants (age two and under) because, in contrast

to the immunity generated by most antigens, a polysaccharide-specific immune response in infants is T-cell-independent. In the absence of T-cell involvement, an immune response is of short duration. More importantly, no memory is demonstrable, so no "booster" reactions occur. Furthermore, antibody affinity maturation does not occur. These deficiencies are due to the inability of polysaccharides to specifically bind to T-cells. Presumably, the structural features required for association with a T-cell receptor do not exist in the majority of polysaccharides. Because of the T-cell independent nature of the immune response, the antibody response to a polysaccharide in infants is limited to antibodies of the IgM isotype; the isotype switching necessary for production of non-IgM antibodies requires T-cell involvement. Polysaccharide antigens present less of a problem in more mature humans (over age two), as they are able to induce antibodies of the IgG isotype as well as IgM (Yount *et al., J. Exp. Med. 127*:633-646 (1968)).

The group B meningococcal polysaccharide is even less immunogenic in humans of all ages than other polysaccharides. Two major explanations have been proposed to account for this characteristic (Jennings, H.J., *Adv. Carbohydr. Chem. Biochem. 41*:155-208 (1983); Lifely, M.R. *et al., Vaccine 5*:11-26 (1987)). One is that the group B meningococcal polysaccharide, an α-(2→8)-linked sialic acid homopolymer, is rapidly depolymerized in human tissue because of the action of neuraminidase. The other is that the structure is recognized as "self" by the human immune system and in consequence, the production of antibody specific for this structure is suppressed. The weight of evidence is in favor of the latter explanation because a neuraminidase-sensitive variant of the group C meningococcal polysaccharide [an α-(2→9)-linked sialic acid homopolymer] still proved to be highly immunogenic in man (Glode, M.P. *et al., J. Infect. Dis. 139*:52-59 (1979)). In addition it was demonstrated that conjugation of the group B polysaccharide to a protein carrier (tetanus toxoid) through its terminal nonreducing sialic acid, which stabilizes the polysaccharide to neuraminidase, did not result in any significant enhancement in its immunogenicity (Jennings, H.J.

and Lugowski, C., *J. Immunol. 127*:1011-1018 (1981)). The above observations are consistent with a theory that the immune mechanism avoids the production of antibody having a specificity for the α-(2→8)-linked sialic acid residues. This theory was further confirmed by the identification of α-(2→8)-linked sialic acid residues in the oligosaccharides of human and animal tissue. A novel approach to overcoming the poor immunogenicity of the group B polysaccharide has been to modify it chemically.

The T-cell independent quality of polysaccharide antigens in infant humans can be overcome by conjugating (covalently coupling) the polysaccharide to a protein carrier. The capsular polysaccharides of the bacteria primarily responsible for postneonatal meningitis have been conjugated to protein carriers: these include type b *H. influenzae* (Schneerson, R. *et al., J. Exp. Med. 152*:361-376 (1980); Anderson, P.W., *Infect. Immun. 39*:233-238 (1983); Marburg, S. *et al., J. Am. Chem. Soc. 108*:5282-5287 (1986)), group A (Jennings, H.J. and Lugowski, C., *J. Immunol. 127*:1011-1018 (1981)) ; Beuvery, E.C. *et al., Vaccine 1*:31-36 (1983)), B (Jennings, H.J. and Lugowski, C., *J. Immunol. 127*:1011-1018 (1981)), and C (Jennings, H.J. and Lugowski, C., *J. Immunol. 127*:1011-1018 (1981)); Beuvery, E.C. *et al., Infect. Immun. 40*:39-45 (1983)) *N. meningitidis*, and type 6A *Strep. pneumoniae* (Chu, C. *et al., Infect. Immun. 40*:245-256 (1983)). For the choice of carrier protein most investigators have used tetanus toxoid or diphtheria toxoid, two proteins currently used as infant vaccines. A recent innovation on this theme has been the use of a mutant-derived diphtheria toxin (CRM_{197}) (Anderson, P.W., *Infect. Immun. 39*:233-238 (1983)) which is nontoxic. The significance of this protein is that because it does not require detoxifying by treatment with formaldehyde, all its amino groups remain underivatized, which greatly facilitates the conjugation process.

The use of other potential bacterial proteins as carriers has not been extensively explored. However, a serotype outer member protein of *N. meningitidis* has been used as a protein carrier (Marburg, S. *et al., J. Am. Chem. Soc. 108*:5282-5287 (1986)).

In light of the foregoing, it will be clear that there is a significant need for a process by which large quantities of the outer membrane group B porin proteins of *N. meningitidis* can be obtained. It will also be clear that a need exists for a polysaccharide vaccine which would give complete coverage to the three major serogroups of *N. meningitidis*, groups A, B and C, and which would provide immunity against these organisms to both infants and more mature humans.

Summary of the Invention

It is a general object of the invention to provide a method of expressing in yeast the meningococcal group B porin proteins, in particular, the class 3 porin protein.

It is a specific object of the invention to provide a method of expressing the outer membrane meningococcal group B porin protein or a fusion protein thereof in yeast, comprising:

(a) cloning into a plasmid having a selectable marker a gene coding for a protein selected from the group consisting of:

(i) a mature porin protein

(ii) a fusion protein which is a mature porin protein fused to a yeast secretion signal peptide;

wherein said gene is operably linked to a yeast promoter;

(b) transforming said plasmid containing said gene into a yeast strain;

(c) selecting the transformed yeast by growing said yeast in a culture medium allowing selection of said transformed yeast;

(d) growing the transformed yeast, and

(e) inducing expression of said protein to give yeast containing said protein;

wherein the protein so expressed comprises more than about 2% of the total protein expressed in said yeast..

It is another specific object of the invention to provide a method of expressing a mature porin protein or fusion protein thereof, wherein the protein so expressed comprises about 3-5% of the total protein expressed in yeast.

It is yet another specific object of the invention to provide a method of expressing a mature porin protein wherein the protein is the *Neisseria meningitidis* outer membrane meningococcal group B porin protein (MB3).

It is another specific object of the invention to provide a method of expressing a mature porin protein or fusion protein thereof, wherein the yeast promoter is the AOX1 promoter.

It is another specific object of the invention to provide a method of expressing the outer membrane meningococcal group B porin protein or a fusion protein thereof in yeast, wherein the yeast secretion signal peptide is selected from the group consisting of the secretion signal of the *S. cerevisiae* α-mating factor prepro-peptide and the secretion signal of the *P. pastoris* acid phosphatase gene (*PHO*).

It is yet another specific object of the invention to provide a method of expressing MB3 or a fusion protein thereof in yeast as described above, wherein the plasmid is selected from the group consisting of pHIL-D2, pHIL-S1, pPIC9 and pPIC9K.

It is a further specific object of the invention to provide a method of expressing the above-described meningococcal group B porin protein or fusion protein wherein at least one codon of the 5′ region of the gene encoding said protein has been changed so as to be optimized for yeast codon usage.

It is still a further specific object of the invention to provide a method of expressing the above-described meningococcal group B porin protein or fusion protein wherein the 5′ region of the gene encoding said protein comprises a nucleotide sequence that incorporates codons optimized for *P. pastoris* codon usage.

It is another specific object of the invention to provide a method as described above wherein the codon changes are selected from the group of

changes consisting of: (GTT to GTC at positions 4-6 of the native sequence), (ACC to ACT at positions 7-9 of the native sequence), (CTG to TTG at positions 10-12 of the native sequence), (GGC to GGT at positions 16-18 of the native sequence), (ACC to ACT at positions 19-21 of the native sequence), (ATC to ATT at positions 22-24 of the native sequence), (AAA to AAG at positions 25-27 of the native sequence), (GCC to GCT at positions 28-30 of the native sequence), (GGC to GGT at positions 31-33 of the native sequence), (GTA to GTT at positions 34-36 of the native sequence), (GAA to GAG at positions 37-39 of the native sequence); wherein said positions are numbered from the first nucleotide of the native nucleotide sequence encoding said protein.

It is another specific object of the invention to provide a method as described above wherein the 5′ region of the gene includes codons optimized for *P. pastoris* codon usage, and wherein the nucleotide sequence is SEQ ID NO: 26.

It is another specific object of the invention to provide a method of expressing the above-mentioned protein wherein the yeast secretes the protein or fusion protein.

It is another specific object of the invention to provide a method of expressing the above-mentioned protein wherein the vector from which the secreted protein is expressed is selected from the group consisting of pHIL-S1, pPIC9, and pPIC9K.

It is another specific object of the invention to provide a method of purifying insoluble, intracellular outer membrane meningococcal group B porin protein or fusion protein thereof obtained according to the invention comprising:

(a) lysing the yeast described above which has expressed the protein to release said protein as an insoluble membrane bound fraction;

(b) washing the insoluble material obtained in step (a) with buffers to remove contaminating yeast cellular proteins;

(c) suspending and dissolving said insoluble fraction obtained in step (b) in aqueous solution of denaturant:

(d) diluting the solution obtained in step (c) with a detergent; and

(e) purifying said protein by gel filtration and ion exchange chromatography.

It is another specific object of the invention to provide a method of purifying the outer membrane meningococcal group B porin protein or fusion protein thereof obtained according to the invention comprising:

(a) centrifuging the yeast culture described above which has expressed the protein to isolate the protein as soluble secreted material;

(b) removing contaminating yeast culture impurities from the soluble secreted material obtained in step (a) by precipitating said impurities with about 20% ethanol, wherein the soluble secreted material remains in the soluble fraction;

(c) precipitating the secreted material from the soluble fraction of step (b) with about 80% ethanol;

(d) washing the precipitated material obtained in step (c) with a buffer to remove contaminating yeast secreted proteins;

(e) suspending and dissolving the precipitated material obtained in step (d) in an aqueous solution of detergent; and

(f) purifying the protein by ion exchange chromatography.

It is a further specific object of the invention to provide a yeast host cell that contains a gene coding for a protein selected from the group consisting of:

(a) a mature porin protein

(b) a fusion protein which is a mature porin protein fused to a yeast secretion signal peptide;

wherein said gene is operably linked to a yeast promoter.

It is still another specific object of the invention to provide a yeast host cell as described above which is capable of expressing the *Neisseria meningitidis* mature outer membrane class 3 protein of serogroup B (MB3).

It is still another specific object of the invention to provide a yeast host cell as described above wherein the yeast promoter is the AOX1 promoter.

It is another object of the invention to provide a vaccine comprising group A meningococcal polysaccharide (GAMP), group B meningococcal polysaccharide (GBMP), and group C meningococcal polysaccharide (GCMP) antigens, together with a pharmaceutically acceptable carrier.

It is still another specific object of the invention to provide a method of inducing an immune response in a mammal, comprising administering to a mammal the above-described vaccine in an amount sufficient to induce an immune response in a mammal.

Further objects and advantages of the present invention will be clear from the description that follows.

Brief Description of the Drawings

Figure 1: A diagram showing the sequencing strategy of the *PorB* gene. The PCR product described in Example 1 (Materials and Methods section) was ligated into the *Bam*HI-*Xho*I site of the expression plasmid pET-17b. The initial double stranded primer extension sequencing was accomplished using oligonucleotide sequences directly upstream of the *Bam*HI site and just downstream of the *Xho*I site within the pET-17b plasmid. Additional sequence data was obtained by making numerous deletions in the 3′ end of the gene, using exonuclease III/mung bean nuclease reactions. After religation and transformation back into *E. coli*, several clones were selected on size of insert and subsequently sequenced. This sequencing was always from the 3′ end of the gene using an oligonucleotide primer just downstream of the *Bpu*11021 site.

Figure 2: A gel electrophoresis showing the products of the PCR reaction (electrophoresed in a 1% agarose using TAE buffer).

Figures 3A and 3B. Fig. 3A: SDS-PAGE analysis of whole cell lysates of *E. coli* hosting the control pET-17b plasmid without inserts and an *E. coli*

clone harboring pET-17b plasmid containing an insert from the obtained PCR product described in the materials and methods section. Both cultures were grown to an O.D. of 0.6 at 600 nm, IPTG added, and incubated at 37°C for 2 hrs. 1.5 mls of each of the cultures were removed, centrifuged, and the bacterial pellet solubilized in 100 µl of SDS-PAGE preparation buffer. Lane A shows the protein profile obtained with 10 µl from the control sample and Lanes B (5 µl) and C (10 µl) demonstrate the protein profile of the *E. coli* host expressing the PorB protein. Fig. 3B: Western blot analysis of whole cell lysates of *E. coli* harboring the control pET-17b plasmid without insert after 2 hrs induction with IPTG, Lane A, 20 µl and a corresponding *E. coli* clone containing a porB-pET-17b plasmid, Lane B, 5 µl; Lane C, 10 µl; and Lane D, 20 µl. The monoclonal antibody 4D11 was used as the primary antibody and the western blot developed as described. The pre-stained low molecular weight standards from BRL were used in each case.

Figure 4: The nucleotide sequence and the translated amino acid sequence of the mature *PorB* gene cloned into the expression plasmid pET-17b. The two nucleotides which differ from the previously published serotype 15 *PorB* are underlined.

Figure 5: A graph showing the Sephacryl S-300 column elution profile of both the wild type Class 3 protein isolated from the meningococcal strain 8765 and the recombinant Class 3 protein produced by BL21(DE3) -Δ*ompA* *E. coli* strain hosting the r3pET-17b plasmid as monitored by absorption at 280nm and SDS-PAGE analysis. The void volume of the column is indicated by the arrow. Fractions containing the meningococcal porin and recombinant porin as determined by SDS-PAGE are noted by the bar.

Figure 6: A graph showing the results of the inhibition ELISA assays showing the ability of the homologous wild type (wt) PorB to compete for reactive antibodies in six human immune sera. The arithmetic mean inhibition is shown by the bold line.

Figure 7: A graph showing the results of the inhibition ELISA assays showing the ability of the purified recombinant PorB protein to compete for reactive antibodies in six human immune sera. The arithmetic mean inhibition is shown by the bold line.

Figure 8: A graph showing a comparison of these two mean inhibitions obtained with the wt and recombinant PorB protein.

Figure 9A and 9B: The nucleotide sequence and the translated amino acid sequence of the mature class II porin gene cloned into the expression plasmid pET-17b.

Figure 10A and 10B: The nucleotide sequence and the translated amino acid sequence of the fusion class II porin gene cloned into the expression plasmid pET-17b.

Figure 11 (panels A and B): Panel A depicts the restriction map of the pET-17b plasmid. Panel B depicts the nucleotide sequence between the *Bgl*II and *Xho*I sites of pET-17b. The sequence provided by the plasmid is in normal print while the sequence inserted from the PCR product are identified in bold print. The amino acids which are derived from the plasmid are in normal print while the amino acids from the insert are in bold. The arrows demarcate where the sequence begins to match the sequence in Figure 4 and when it ends.

Figure 12: A graph showing the level of expression of MB3 for clone pnv 322, where the expression vector used is pHIL-D2. The level of MB3 expressed is depicted as mg of insoluble MB3 per gram of cell pellet per unit time.

Figure 13A: The DNA sequence and translated amino acid sequence of pNV15 (MB3 in pET24a) before codon preference optimization.

Figure 13B: The DNA sequence and translated amino acid sequence of Men.Class3 opt. (MB3 optimized for yeast codon preference).

Figures 14A and 14B: Graphs showing the elution of MB3 from a size exclusion column. MB3 expressed in an intracellular form was purified by a denaturation/renaturation protocol, followed by gel filtration and ion exchange chromatography. The resultant purified protein exhibited by size exclusion

chromatography an elution profile which resembles the recombinant class 3 protein overexpressed in *E. coli*, and both give the same elution profile as the native wild-type counterpart. This indicates that MB3 refolds and oligomerizes, achieving full native conformation. 14(A): the elution profile of MB3; 14(B): the elution profile of class 3 protein expressed and refolded from *E. coli* inclusion bodies.

Figure 15: A graph showing the size exclusion chromatography of purified MB3 on a Superose 12 (Pharmacia) column connected to an HPLC (Hewlett Packard model 1090). Based on the comparison of MB3 with the native wild-type counterpart, as well as calibration using molecular weight standards (designated as arrows), the elution profile is indicative of trimeric assembly. Molecular weight markers are: 1 = thyroglobulin (670,000); 2 = gammaglobulin (158,000); 3 = myoglobin (17,000).

Figures 16A, 16B and 16C: The DNA sequence of clone pnv 322. This clone has the MB3 gene inserted into the *EcoR*I site of the Invitrogen expression vector pHIL-D2. MB3 is thus inserted directly downstream from the *AOX1* promoter. This construct allows intracellular expression. Vector sequences are shown in upper case letters, while the MB3 sequence is given in lower case letters.

Figures 17A, 17B and 17C: The DNA sequence of clone pnv 318. This clone has the MB3 gene inserted into the *XhoI-BamHI* sites of the Invitrogen expression vector pHIL-S1. MB3 is thus inserted directly downstream from the *PHO1* leader peptide, in frame with the secretion signal open reading frame for secretion of expressed protein. Vector sequences are shown in upper case letters, while the MB3 sequence is given in lower case letters.

Figures 18A, 18B and 18C: The DNA sequence of clone pnv 342. This clone has the MB3 gene inserted into the *EcoR*I-*AvrII* sites of the Invitrogen expression vector pPIC-9. MB3 is thus inserted directly downstream from the secretion signal of α-factor prepro peptide, for secretion of expressed protein.

Vector sequences are shown in upper case letters, while the MB3 sequence is given in lower case letters.

Figures 19A, 19B and 19C: The DNA sequence of clone pnv 350. This clone has the MB3 gene inserted into the *EcoRI-AvrII* sites of the Invitrogen expression vector pPIC-9K. MB3 is thus inserted directly downstream from the secretion signal of α-factor prepro peptide, for secretion of expressed protein. Vector sequences are shown in upper case letters, while the MB3 sequence is given in lower case letters.

Figure 20: A graph showing the absorbance spectra (electropherogram) of GAMP, TT-monomer, and GAMP-TT conjugate.

Figure 21: A graph showing the absorbance spectra (electropherogram) of GCMP, TT-monomer, and GCMP-TT conjugate.

Figure 22: A graph showing the A-specific IgG ELISA titer elicited by monovalent (A) and trivalent (A/B/C) meningococcal conjugate vaccines in mice.

Figure 23: A graph showing the B-specific IgG ELISA titer elicited by monovalent (A) and trivalent (A/B/C) meningococcal conjugate vaccines in mice.

Figure 24: A graph showing the C-specific IgG ELISA titer elicited by monovalent (C) and trivalent (A/B/C) meningococcal conjugate vaccines in mice.

Figure 25: A graph showing the A-specific bacteriocidal activity elicited by monovalent (A) and trivalent (A/B/C) meningococcal conjugate vaccines in mice.

Figure 26: A graph showing the B-specific bacteriocidal activity elicited by monovalent (A) and trivalent (A/B/C) meningococcal conjugate vaccines in mice.

Figure 27: A graph showing the C-specific bacteriocidal activity elicited by monovalent (A) and trivalent (A/B/C) meningococcal conjugate vaccines in mice.

Detailed Description of the Invention

It is possible to overcome some of the difficulties involved in expressing and purifying the outer membrane group B porin proteins of *N. meningitidis* from *E. coli*. The DNA sequences of the mature porin proteins, e.g. class 2 and class 3 as well as fusions thereof, were amplified using the chromosome of the meningococcal bacteria as a template for the PCR reaction. The amplified porin sequences were ligated and cloned into an expression vector containing the T7 promoter. *E. coli* strain BL21 lysogenic for the DE3 lambda phage (Studier and Moffatt, *J. Mol. Biol. 189*:113-130 (1986)), modified to eliminate the *ompA* gene, was selected as one expression host for the pET-17b plasmid containing the porin gene. Upon induction, large amounts of the meningococcal porin proteins accumulated within *E. coli* without any obvious lethal effects to the host bacterium. The expressed meningococcal porin proteins were extracted and processed through standard procedures and finally purified by molecular sieve chromatography and ion exchange chromatography. As judged by the protein profile from the molecular sieve chromatography, the recombinant meningococcal porins eluted from the column as trimers. To be certain that no PCR artifacts had been introduced into the meningococcal porin genes to allow for such high expression, the inserted PorB gene sequence was determined. Inhibition ELISA assays were used to give further evidence that the expressed recombinant porin proteins had renatured into their natural antigenic and trimer conformation.

As an alternative to the bacterial *E. coli* host system, Meningococcal B Class 3 porin protein (MB3) may be expressed in yeast. A preferred host is the methylotrophic yeast *Pichia pastoris*, which may be transformed with the *Pichia* Expression Kit developed by Invitrogen. Yeasts are attractive hosts for the production of heterologous proteins. Unlike prokaryotic systems, their eukaryotic subcellular organization enables them to carry out many of the post-translational

folding, processing and modification events required to produce "authentic" and bioactive proteins. As a eukaryote, *Pichia pastoris* has many of the advantages of a higher eukaryotic expression system, while being as easy to manipulate as *E. coli* or *Saccharomyces cerevisiae*. As a yeast, it shares the advantages of molecular and genetic manipulations with *Saccharomyces*, and it has the added advantages of 10- to 100-fold higher heterologous protein expression levels and the protein processing characteristics of higher eukaryotes.

Expression in *Pichia* also provides advantages compared to expression in other yeast strains because *Pichia* does not tend to hyperglycosylate proteins as does *S. cerevisiae*. Further, proteins expressed and modified in *Pichia* may be more useful therapeutically than those produced by *S. cerevisiae*, as oligosaccharides added by *Pichia* lack the α1,3 glycan linkages which are believed to be primarily responsible for the hyper-antigenic nature of proteins produced by *S. cerevisiae*. Several vaccine antigens have been produced in yeast cells, including hepatitis B surface antigen which is in clinical use (Cregg *et al.*, *Bio/Technology 11*:905-910 (1993)).

Unlike the porin proteins of *E. coli* and a few other gram negative bacteria, relatively little is known about how changes in the primary sequence of porins from *Neisseria* effect their ion selectivity, voltage dependence, and other biophysical functions. Recently, the crystalline structure of two *E. coli* porins, OmpF and PhoE, were solved to 2.4Å and 3.0Å, respectively (Cowan, S.W., *et al.*, *Nature 358*:727-733 (1992)). Both of these *E. coli* porins have been intensively studied owing to their unusual stability and ease with which molecular genetic manipulations could be accomplished. The data obtained for the genetics of these two porins correlated well with the crystalline structure. Although it has been shown in several studies using monoclonal antibodies to select neisserial porins that the surface topology of *Neisseria* closely resembles that of these two *E. coli* porins (van der Ley, P., *et al.*, *Infect. Immun. 59*:2963-2971 (1991)), almost no information is available about how changes in amino acid sequences in specific areas of the neisserial porins effect their biophysical characteristics,

as is known about the *E. coli* porins (Cowan, S.W., *et al.*, *Nature 358*:727-733 (1992)).

Two of the major problems impeding this research are: (1) the inability to easily manipulate *Neisseria* genetically by modern molecular techniques and (2) the inability to express sufficient quantities of neisserial porins in *E. coli* or yeast for further purification to obtain biophysical and biochemical characterization data. In fact, most of the DNA sequence data on gonococcal and meningococcal porins have been obtained by cloning overlapping pieces of the porin gene and then reconstructing the information to reveal the entire gene sequence (Gotschlich, E.C., *et al.*, *Proc. Natl. Acad. Sci. USA 84*:8135-8139 (1987); Murakami, K., *et al.*, *Infect. Immun. 57*:2318-2323 (1989)). Carbonetti *et al.* were the first to clone an entire gonococcal porin gene into *E. coli* using a tightly controlled pT7-5 expression plasmid. The results of these studies showed that when the porin gene was induced, very little porin protein accumulated and the expression of this protein was lethal to the *E. coli* (Carbonetti and Sparling, *Proc. Natl. Acad. Sci. USA 84*:9084-9088 (1987)). In additional studies, Carbonetti *et al.* (*Proc. Natl. Acad. Sci. USA 85*:6841-6845 (1988)) did show that alterations in the gonococcal porin gene could be made in this system in *E. coli* and then reintroduced into gonococci. However, the ease with which one can make these manipulations and obtain enough porin protein for further biochemical and biophysical characterization seems limited.

Feavers *et al.* have described a method to amplify, by PCR, neisserial porin genes from a wide variety of sources using two synthesized oligonucleotides to common domains at the 5′ and 3′ ends of the porin genes respectively (Feavers, I.M., *et al.*, *Infect. Immun. 60*:3620-3629 (1992)). The oligonucleotides were constructed such that the amplified DNA could be forced cloned into plasmids using the restriction endonucleases *Bgl*II and *Xho*I.

Using the Feavers *et al.* PCR system, the DNA sequence of the mature PorB protein from meningococcal strain 8765 serotype 15 was amplified and ligated into the *Bam*HI-*Xho*I site of the T7 expression plasmid pET-17b. This

placed the mature PorB protein sequence in frame directly behind the T7 promoter and 20 amino acids of the ϕ10 protein including the leader sequence. Upon addition of IPTG to a culture of *E. coli* containing this plasmid, large amounts of PorB protein accumulated within the bacteria. A complete explanation for why this construction was non-lethal to the *E. coli* and expressed large amount of the porin protein, await further studies. However, one possible hypothesis is that by replacing the neisserial promoter and signal sequence with that of the T7 and ϕ10 respectively, the porin product was directed to the cytoplasm rather than toward the outer membrane. Henning and co-workers have reported that when *E. coli* OmpA protein and its fragments are expressed, those products which are found in the cytoplasm are less toxic than those directed toward the periplasmic space (Klose, M., *et al.*, *J. Biol. Chem. 263*:13291-13296 (1988); Klose, M., *et al.*, *J. Biol. Chem. 263*:13297-13302 (1988); Freudl, R., *et al.*, *J. Mol. Biol. 205*:771-775 (1989)). Whatever the explanation, once the PorB protein was expressed, it was easily isolated, purified and appeared to reform into trimers much like the native porin. The results of the inhibition ELISA data using human immune sera suggests that the PorB protein obtained in this fashion regains most if not all of the antigenic characteristics of the wild type PorB protein purified from meningococci. This expression system lends itself to the easy manipulation of the neisserial porin gene by modern molecular techniques. In addition, this system allows one to obtain large quantities of pure porin protein for characterization. In addition, the present expression system allows the genes from numerous strains of *Neisseria*, both gonococci and meningococci, to be examined and characterized in a similar manner.

The *Neisseria meningitidis* outer membrane class 3 protein from serogroup B (MB3) was also expressed in the methylotrophic yeast *Pichia pastoris* by placing the MB3 DNA fragment under the control of the strong *P. pastoris* alcohol oxidase promoter *AOX1*. Upon induction on methanol, strains of *P. pastoris* transformed with the recombinant plasmids produced either a native or a fusion MB3 protein, which were reactive with mouse polyclonal

antibodies raised against the wild type counterpart. In shaking flask cultures, engineered *P. pastoris* yielded about 1-3 mg of expressed protein per gram of pelleted wet cells, or 100-600 mg per liter, which corresponded to 10-15% of the yeast cell suspension or about 3-5% of total cellular proteins (Table 4). Full-length MB3 DNA was cloned into each of four Pichia Expression Vectors developed by Invitrogen. To obtain the expression of monomeric, full size 34 kDa MB3 protein, the intracellular pHIL-D2 vector was used. A map of the pHIL-D2 vector may be found on p. 19 of the Invitrogen Instruction Manual for the *Pichia* Expression Kit, Version E, the contents of which is hereby incorporated by reference. This construct provided maximal expression levels (up to 3 mg of MB3 per gram of cells) (Tables 3 and 4). The expressed product was not secreted, being mainly (95%) insoluble, and it was tightly associated with cell membranes.

To further increase the possibility for the secretion of expressed MB3, three other vectors with different secretion signals were also used: the vector pHIL-S1, which carries a native *Pichia pastoris* signal sequence from the acid phosphatase gene, *PHO1*, and the vectors pPIC9 and pPIC9K, which carry the secretion signal from the *S. cerevisiae* α-mating factor prepro-peptide. Maps of the pHIL-S1 and pPIC9 vectors may be found on pp. 21-22 of the Invitrogen Instruction Manual for the *Pichia* Expression Kit, Version E. It was found that the pHIL-S1/MB3 construct provided the expression of a MB3- PHO1 fusion polypeptide with an apparent molecular weight of 36.5 kDa, which was partly processed to generate mature 34 kDa MB3. About 5-10% of expressed MB3 was secreted to the yeast growth medium, and about 40-50% of the 36.5 kDa fusion polypeptide was cleaved (Table 4). *Pichia* recombinants transformed by pPIC9/MB3 or pPIC9K/MB3 constructs expressed only MB3 fused with α-factor, yielding a fusion polypeptide of approximately 45 kDa. There was no evidence of any cleavage or processing of that fusion protein.

Preliminary studies on the isolation and purification of recombinant MB3 (pHIL-D2/MB3 containing transformants) suggest that when expressed in *P.*

pastoris, MB3 may form trimers under native conditions, and that the native protein is resistant to trypsin digestion. These results are similar to those which have been observed for the wild-type counterpart.

An increase in the yield of expressed MB3 may be obtained by using strains of *Pichia* which contain multiple copies of the MB3 expression cassette. Strains harboring multiple copies exist naturally within transformed cell populations at <10% frequency. These strains may be identified either by directly screening large numbers of transformants for levels of MB3 expression via SDS-PAGE or immunoblotting, or indirectly screening by "dot blot" hybridization to select for clones containing multiple copies of the MB3 gene (Cregg *et al.*, *Bio/Technology 11*:905-910 (1993)). Alternatively, such multiple integrated clones may be constructed by using a new pAO815 vector (Invitrogen), which allows cloning of multiple copies of the gene of interest via repeated cassette insertion steps (*Ibid.* at p. 907). Scale-up procedures using a fermenter will provide higher yeast cell densities and therefore improve the yields of the expressed proteins by at least 5-10 times. Optimization of protein expression (*i.e.*, growth media composition, buffering capacity, casamino acids supplementation, increase of methanol concentration, etc.) may be carried out with routine experimentation.

Another way to identify *Pichia* transformants having multiple copies of MB3 takes advantage of the fact that the *Pichia* expression vector pPIC9K carries the kanamycin resistance gene which confers resistance to G418; in other respects, pPIC9K corresponds to pPIC9. Spontaneous generation of multiple insertion events can then be identified by the level of resistance to G418. *Pichia* transformants are selected on histidine-deficient medium and screened for their level of resistance to G418. An increased level of resistance to G418 indicates multiple copies of the kanamycin resistance gene.

Thus, the present invention relates to a method of expressing an outer membrane meningococcal group B porin protein, in particular, the class 2 and class 3 porin proteins.

In one embodiment, the present invention relates to a method of expressing the outer membrane meningococcal group B porin protein in *E. coli* comprising:

(a) transforming *E. coli* by a vector comprising a selectable marker and a gene coding for a protein selected from the group consisting of:

(i) a mature porin protein, and

(ii) a fusion protein comprising a mature porin protein fused to amino acids 1 to 20 or 22 of the T7 gene ϕ10 capsid protein;

wherein said gene is operably linked to the T7 promoter;

(b) growing the transformed *E. coli* in a culture media containing a selection agent, and

(c) inducing expression of said protein;

wherein the protein so produced comprises more than about 2% of the total protein expressed in the *E. coli*.

In a preferred embodiment, the meningococcal group B porin protein or fusion protein expressed comprises more than about 5% of the total proteins expressed in *E. coli*. In another preferred embodiment, the meningococcal group B porin protein or fusion protein expressed comprises more than about 10% of the total proteins expressed in *E. coli*. In yet another preferred embodiment, the meningococcal group B porin protein or fusion protein expressed comprises more than about 30% of the total proteins expressed in *E. coli*.

Examples of plasmids which contain the T7 inducible promotor include the expression plasmids pET-17b, pET-11a, pET-24a-d(+) and pET-9a, all of which are commercially available from Novagen (565 Science Drive, Madison, WI 53711). These plasmids comprise, in sequence, a T7 promoter, optionally a lac operator, a ribosome binding site, restriction sites to allow insertion of the structural gene and a T7 terminator sequence. *See*, the Novagen catalogue, pages 36-43 (1993).

In a preferred embodiment, *E. coli* strain BL21 (DE3) Δ*omp*A is employed. The above mentioned plasmids may be transformed into this strain or the wild-type strain BL21(DE3). *E. coli* strain BL21 (DE3) Δ*omp*A is preferred as no OmpA protein is produced by this strain which might contaminate the purified porin protein and create undesirable immunogenic side effects.

The transformed *E. coli* are grown in a medium containing a selection agent, e.g. any β-lactam to which *E. coli* is sensitive such as ampicillin. The pET expression vectors provide selectable markers which confer antibiotic resistance to the transformed organism.

High level expression of meningococcal group B porin protein can be toxic in *E. coli*. Surprisingly, the present invention allows *E. coli* to express the protein to a level of at least almost 30% and as high as >50% of the total cellular proteins.

In another embodiment, the present invention relates to a method of expressing an outer membrane meningococcal group B porin protein in yeast comprising:

(a) ligating into a plasmid having a selectable marker a gene coding for a protein selected from the group consisting of :

(i) a mature porin protein, and

(ii) a fusion protein comprising a mature porin protein fused to a yeast secretion signal peptide;

wherein said gene is operably linked to a yeast promoter;

(b) transforming the plasmid containing the gene into a yeast strain;

(c) selecting the transformed yeast by growing said yeast in a culture medium allowing selection of said transformed yeast;

(d) growing the transformed yeast, and

(e) inducing expression of said protein to give yeast containing said protein.

Transformation of the yeast host may be accomplished by any one of several techniques that are well known by those of ordinary skill in the art. These

techniques include direct or liposome-mediated transformation of yeast cells whose cell wall has been partially or completely destroyed to form spheroplasts, treatment of the host with alkali cations and PEG, and freeze-thawing combined with PEG treatment. (*See* Weber *et al.*, *Nonconventional Yeasts: Their Genetics and Biotechnological Applications*, *CRC Crit. Rev. Biotechnol.* 7: 281, 317 (1988) and the references cited therein, all of which are hereby fully incorporated by reference.)

In another preferred embodiment, the mature porin protein or fusion protein expressed comprises more than about 2% of the total protein expressed in the yeast host. In yet another preferred embodiment, the mature porin protein or fusion protein expressed comprises about 3-5% of the total protein expressed in the yeast host.

In another preferred embodiment, the mature porin protein is the *Neisseria meningitidis* mature outer membrane class 3 protein from serogroup B.

In another preferred embodiment, the present invention relates to performing the above method of expressing the outer membrane meningococcal group B porin protein or fusion protein in yeast, wherein said yeast is selected from the group consisting of: *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Saccharomyces uvarum*, *Saccharomyces carlsbergensis*, *Saccharomyces diastaticus*, *Candida tropicalis*, *Candida maltosa*, *Candida parapsilosis*, *Pichia pastoris*, *Pichia farinosa*, *Pichia pinus*, *Pichia vanrijii*, *Pichia fermentans*, *Pichia guilliermondii*, *Pichia stipitis*, *Saccharomyces telluris*, *Candida utilis*, *Candida guilliermondii*, *Hansenula henricii*, *Hansenula capsulata*, *Hansenula polymorpha*, *Hansenula saturnus*, *Lypomyces kononenkoae*, *Kluyveromyces marxianus*, *Candida lipolytica*, *Saccaromycopsis fibuligera*, *Saccharomycodes ludwigii*, *Saccharomyces kluyveri*, *Tremella mesenterica*, *Zygosaccharomyces acidofaciens*, *Zygosaccharomyces fermentati*, *Yarrowia lipolytica*, and *Zygosaccharomyces soja*. Many of these yeast hosts are available from the American Type Culture Collection, Rockville, Md.

In another preferred embodiment, the nucleotide sequence of the gene encoding the mature porin protein or fusion protein incorporates codons which are optimized for yeast codon usage. In yet another preferred embodiment, the nucleotide sequence of the gene encoding the mature porin protein which has been optimized for yeast codon usage is the nucleotide sequence SEQ ID NO: 26.

In another preferred embodiment, the yeast secretion signal peptide is selected from the group consisting of the secretion signal of the *S. cerevisiae* α-mating factor prepro-peptide and the secretion signal of the *P. pastoris* acid phosphatase gene.

In another preferred embodiment, the yeast secretes the protein or fusion protein.

In another preferred embodiment, the yeast promoter to which the gene is operably linked is selected from a group consisting of the AOX1 promoter, the GAPDH promoter, the PHO5 promoter, the glyceraldehyde-3-phosphate dehydrogenase (TDH3) promoter, the ADH1 promoter, the MFα1 promoter, and the GAL10 promoter. Examples of plasmids which contain the AOX1 promoter include the expression plasmids pHIL-D2, pHIL-S1, pPIC9, and pPIC9K. These plasmids comprise, in sequence, an AOX1 promoter, restriction sites to allow insertion of the structural gene, an AOX1 transcription termination fragment, an open reading frame encoding HIS4 (histidinol dehydrogenase), an ampicillin resistance gene, and a ColE1 origin. In addition, plasmids pPIC9 and pPIC9K comprise the α-factor secretion signal of *S. cerevisiae*, and plasmid pHIL-S1 comprises the *PHO1* secretion signal of *P. pastoris*. pPIC9K also includes the kanamycin resistance gene, which confers resistance to G418 in *Pichia*. The level of G418 resistance in *Pichia* transformants can be used to identify cells which have undergone multiple insertion events. This occurs at a frequency of 1-10%. An increased level of resistance to G418 indicates the presence of multiple copies of the kanamycin resistance gene and of the gene of interest. *See* the Novagene catalogue, Version E, pp. 19-22 (1995).

In another preferred embodiment, yeast host strains having a mutation in a suitable marker gene which causes the strain to have specific nutritional requirements are employed. Expression plasmids carrying a functional copy of the mutated gene as well as a copy of the meningococcal group B porin protein or fusion protein are then transformed into the yeast host strain, and transformants are selected on the basis of their ability to grow on medium lacking the required nutrient. Examples of suitable marker genes, followed by their *S. cerevisiae* notation, include the genes encoding imidazole glycerol phosphate dehydrogenase (*HIS3*), beta-isopropylmalate dehydrogenase (*LEU2*), tryptophan synthase (*TRP5*), argininosuccinate lyase (*ARG4*), *N*-(5'-phosphorilosyl) anthranilate isomerase (*TRP1*), histidinol dehydrogenase (*HIS4*), orotidine-5-phosphate decarboxylase (*URA3*), dihydroorotate dehydrogenase (*URA1*), galactokinase (*GAL1*), and alpha-aminodipate reductase (*LYS2*). After transformed yeast host cells are selected on the basis of their ability to grow in medium lacking the appropriate nutrient, the cells are screened for integration of the meningococcal group B porin protein or fusion protein at the correct loci. This screening is performed by methods well known to those of ordinary skill in the art, for example, by selecting for transformants which grow slowly on medium which lacks the nutrient used to confirm transformation and includes methanol in order to induce expression of the outer membrane meningococcal group B porin protein or fusion protein from the AOX1 promoter. These transformants are then grown up in glycerol-containing medium, and expression of the meningococcal group B porin protein or fusion protein is then induced by the addition of methanol.

In a more preferred embodiment, *P. pastoris* host strains GS115 or KM71 are employed. These strains have a mutation in the histidinol dehydrogenase gene (*his4*) which prevents them from synthesizing histidine. The expression plasmids pHIL-D2, pHIL-S1, pPIC9, and pPIC9K carry the *HIS4* gene which complements *his4* in the host, allowing selection of transformants on histidine-deficient medium. After transformed *P. pastoris* host cells are selected in

histidine-deficient medium, the cells are screened for integration of the meningococcal group B porin protein or fusion protein at the correct loci by selecting for transformants which grow slowly on his$^-$, methanol$^+$ plates. These transformants, which become mutated at the *AOX1* locus when the MB3 gene inserts into the host genome, are only capable of slow growth on methanol, as they are metabolizing methanol with the less efficient *AOX2* gene product. The transformants are then grown up in glycerol-containing medium, and expression of the meningococcal group B porin protein or fusion protein is then induced by the addition of methanol.

In a most preferred embodiment, the present invention relates to performing the above method of expressing the outer membrane meningococcal group B porin protein in yeast, wherein said yeast is *Pichia pastoris*.

In another preferred embodiment, the present invention relates to a vaccine for inducing an immune response in an animal comprising the outer membrane meningococcal group B porin protein or fusion protein thereof, produced according to the above-described methods, together with a pharmaceutically acceptable diluent, carrier, or excipient, wherein the vaccine may be administered in an amount effective to elicit an immune response in an animal to *Neisseria meningitidis*. In a preferred embodiment, the animal is selected from the group consisting of humans, cattle, pigs, sheep, and chickens. In another preferred embodiment, the animal is a human.

In another preferred embodiment, the present invention relates to the above-described vaccine, wherein said outer membrane meningococcal group B porin protein or fusion protein thereof is conjugated to a meningococcal group B capsular polysaccharide (CP). Such capsular polysaccharides may be prepared as described in Ashton, F.E. *et al., Microbial Pathog. 6*:455-458 (1989); Jennings, H.J. *et al., J. Immunol. 134*:2651 (1985); Jennings, H.J. *et al., J. Immunol. 137*:1708-1713 (1986); Jennings, H.J. *et al., J. Immunol. 142*:3585-3591 (1989); Jennings, H.J., "Capsular Polysaccharides as Vaccine Candidates,"

in *Current Topics in Microbiology and Immunology*, *150*:105-107 (1990); the contents of each of which are fully incorporated by reference herein.

The invention also relates to a vaccine capable of simultaneously inducing an immune response against any one of several *N. meningitidis* serogroups. Thus, in another preferred embodiment, the invention relates to a trivalent vaccine comprising the capsular polysaccharides from each of three different serogroups of *N. meningitidis*. Specifically, the vaccine of the invention comprises group A meningococcal polysaccharide (GAMP), group B meningococcal polysaccharide (GBMP), and group C meningococcal polysaccharide (GCMP) antigens, together with a pharmaceutically acceptable carrier.

In a preferred embodiment, group A meningococcal polysaccharide (GAMP), group B meningococcal polysaccharide (GBMP), and group C meningococcal polysaccharide (GCMP) antigens are each conjugated to a protein carrier, thus yielding GAMP, GCMP and GBMP polysaccharide antigen conjugates.

Of course, it will be understood by those of ordinary skill that a number of carrier proteins will be suitable to be used in the polysaccharide-protein conjugates included in the vaccine of the invention. A suitable carrier protein will be one which is safe for administration to mammals, and which is immunologically effective as a carrier. Safety includes absence of primary toxicity and minimal risk of allergic complications.

In general, any heterologous protein could serve as a carrier antigen. The protein may be, for example, native toxin or detoxified toxin (also termed toxoid). In addition, genetically altered proteins which are antigenically similar to toxins and yet non-toxic may be produced by mutational techniques well-known to those of skill in the art. Such an altered toxin is termed a "cross reacting material," or CRM. CRM_{197} is noteworthy, because it differs from native diphtheria toxin at only one amino acid residue, and is immunologically indistinguishable from the native toxin (Anderson, P.W., *Infect. Immun. 39*:233-238 (1983)).

It will be understood by those of skill in the art that the polysaccharide-protein carrier conjugates of the vaccine may be produced by several different methods. The types of covalent bonds which couple a polysaccharide to a protein carrier, and the means of producing them, are well known to those of skill in the art. Details concerning the chemical means by which the two moieties can be linked may be found in U.S. Patent No. 5,371,197, and 4,902,506, the contents of which are herein incorporated by reference in their entirety. One such method is the reductive amination process described in Schwartz and Gray (*Arch. Biochim. Biophys. 181*:542-549 (1977)). This process involves reacting the reducing capsular polysaccharide fragment and bacterial toxin or toxoid in the presence of cyanoborohydride ions, or another reducing agent. Such a process will not adversely affect the toxin or toxoid or the capsular polysaccharide (U.S. Patent No. 4,902,506). Such a conjugation process is also described in Examples 12-14, below.

While tetanus and diphtheria toxins are the prime candidates for carrier proteins, owing to their history of safety, there may be overwhelming reasons, well known to those of ordinary skill in the art, to use another protein. For example, another protein may be more effective as a carrier, or production economics may be significant. Other candidates include toxins or toxoids of pseudomonas, staphylococcus, streptococcus, pertussis and entertoxigenic bacteria, including *Escherichia coli*. A preferred carrier protein to which the group B meningococcal polysaccharide may be conjugated is the class 3 porin protein (PorB) of group B *N. meningitidis*. A preferred protein carrier protein to which GAMP antigen and GCMP antigen may be conjugated is tetanus toxoid.

It is known in the art that the immunogenicity of GBMP is limited in humans, and especially in infant humans, and that direct covalent couplings of the group B polysaccharide to tetanus toxoid yielded a conjugate which failed to induce a significant polysaccharide-specific response in either rabbits (Jennings, H.J. and Lugowski, C., *J. Immunol. 127*:1011-1018 (1981)) or mice (Jennings,

H.J. *et al., J. Immunol. 137*:1708-1713 (1986)). This failure prompted interest in the direct chemical modification of the group B polysaccharide. This was done with the idea of creating synthetic epitopes capable of modulating the immune response in such a way as to produce enhanced levels of cross-reactive B polysaccharide-specific antibodies (Jennings, H.J. *et al., J. Immunol. 137*:1708-1713 (1986)).

It will be understood by those of ordinary skill in the art that in selecting possible chemical modifications of the group B polysaccharide (Jennings, H.J. *et al., J. Immunol. 137*:1708-1713 (1986)), two factors should be considered. First, the chemical modification should be able to be accomplished with facility and with the minimum of degradation of the polysaccharide. Second, in order to produce cross-reactive B polysaccharide-specific antibodies, the antigenicity of the modified polysaccharide to B polysaccharide-specific antibodies should be preserved. It will be understood by those of skill in the art that the ideal chemical modification of group B polysaccharide will retain both the carboxylate and the N-carbonyl groups (Jennings, H.J. *et al., J. Immunol. 137*:1708-1713 (1986)). The most preferred modification which satisfies the above criteria is a modification wherein the N-acetyl groups of the sialic acid residues of the B polysaccharide are removed by strong base and replaced by N-propionyl groups (see Examples 6 and 14).

In a more preferred embodiment, the N-propionylated GBMP is subsequently conjugated to a carrier protein. While any carrier protein which enhances the immunogenicity of N-propionylated GBMP may be used, a preferred protein carrier is the class 3 outer membrane protein of group B *N. meningitidis* (MB3, or PorB).

Thus, in still another preferred embodiment, GBMP antigen is conjugated to PorB after having been N-propionylated.

Preferably, the capsular polysaccharide (CP), which may be group A, B or C meningococcal polysaccharide, is isolated according to Frasch, C.E., "Production and Control of *Neisseria meningitidis* Vaccines" in *Bacterial*

Vaccines, Alan R. Liss, Inc., pages 123-145 (1990), the contents of which are fully incorporated by reference herein, as follows:

Grow organisms in modified Franz medium 10 to 20 hrs

↓ Heat kill, 55°C, 10 min

Remove inactivated cells by centrifugation

↓ Add Cetavlon to 0.1%

Precipitate CP from culture broth

↓ Add calcium chloride to 1 M

Dissolve CP then centrifuge to remove cellular debris

↓ Add ethyl alcohol to 25%

Remove precipitated nucleic acids by centrifugation

↓ Add ethyl alcohol to 80%

Precipitate crude CP and remove alcohol

The crude CP is then further purified by gel filtration chromatography after partial depolymerization with dilute acid, e.g. acetic acid, formic acid, and trifluoroacetic acid (0.01-0.5 N), to give a mixture of polysaccharides having an average molecular weight of 10,000-20,000. Where the CP is GBMP, purified GBMP is then N-deacetylated with NaOH in the presence of sodium borohydride and N-propionylated to afford N-Pr GBMP. Thus, the CP that may be employed in the conjugate vaccines of the present invention may be CP fragments, N-deacylated CP and fragments thereof, as well as N-Pr CP and fragments thereof, so long as they induce active immunity when employed as part of a CP-porin protein conjugate (see Examples 6 and 14).

In a further preferred embodiment, the present invention relates to a method of preparing a polysaccharide conjugate comprising: obtaining the above-described outer membrane meningococcal group B porin protein or fusion protein thereof; obtaining a CP from a *Neisseria meningitidis* organism; and conjugating the protein to the CP.

The conjugates of the invention may be formed by reacting the reducing end groups of the CP to primary amino groups of the porin by reductive amination. The reducing groups may be formed by selective hydrolysis or specific oxidative cleavage, or a combination of both. Preferably, the CP is conjugated to the porin protein by the method of Jennings *et al.*, U.S. Patent No. 4,356,170, the contents of which are fully incorporated by reference herein, which involves controlled oxidation of the CP with periodate followed by reductive amination with the porin protein.

The vaccine of the present invention comprises the meningococcal group B porin protein, fusion protein or conjugate vaccine, or the trivalent GAMP. GBMP and GCMP vaccine, in an amount effective depending on the route of administration. Although subcutaneous or intramuscular routes of administration are preferred, the meningococcal group B porin protein, fusion protein or vaccine of the present invention can also be administered by an intraperitoneal or intravenous route. One skilled in the art will appreciate that the amounts to be administered for any particular treatment protocol can be readily determined without undue experimentation. Suitable amounts might be expected to fall within the range of 2 micrograms of the protein per kg body weight to 100 micrograms per kg body weight.

Thus, in a preferred embodiment, the vaccine comprises about 2 μg of the GAMP, GCMP and GBMP polysaccharide antigen conjugates.

In another preferred embodiment, the vaccine comprises about 5 μg of the GAMP, GCMP and GBMP polysaccharide antigen conjugates.

In yet another referred embodiment, the vaccine comprises about 2 μg of the GAMP and GCMP polysaccharide antigen conjugates, and about 5 μg of the GBMP polysaccharide antigen conjugate.

The vaccine of the present invention may be employed in such forms as capsules, liquid solutions, suspensions or elixirs for oral administration, or sterile liquid forms such as solutions or suspensions. Any inert carrier is preferably used, such as saline, phosphate-buffered saline, or any such carrier in which the

meningococcal group B porin protein, fusion protein or conjugate vaccine have suitable solubility properties. The vaccines may be in the form of single dose preparations or in multi-dose flasks which can be used for mass vaccination programs. Reference is made to Remington's *Pharmaceutical Sciences*, Mack Publishing Co., Easton, PA, Osol (ed.) (1980); and *New Trends and Developments in Vaccines*, Voller *et al.* (eds.), University Park Press, Baltimore, MD (1978), for methods of preparing and using vaccines.

The vaccines of the present invention may further comprise adjuvants which enhance production of porin-specific antibodies. Such adjuvants include, but are not limited to, various oil formulations such as Freund's complete adjuvant (CFA), stearyl tyrosine (ST, *see* U.S. Patent No. 4,258,029), the dipeptide known as MDP, saponin, aluminum hydroxide, and lymphatic cytokine.

Freund's adjuvant is an emulsion of mineral oil and water which is mixed with the immunogenic substance. Although Freund's adjuvant is powerful, it is usually not administered to humans. Instead, the adjuvant alum (aluminum hydroxide) or ST may be used for administration to a human. The meningococcal group B porin protein or a conjugate vaccine thereof may be absorbed onto the aluminum hydroxide from which it is slowly released after injection. The meningococcal group B porin protein or group A, B and C meningococcal polysaccharide conjugate vaccine may also be encapsulated within liposomes according to Fullerton, U.S. Patent No. 4,235,877.

In another preferred embodiment, the present invention relates to a method of inducing an immune response in an animal comprising administering to the animal the vaccine of the invention, produced according to methods described, in an amount effective to induce an immune response.

In a further embodiment, the invention relates to a method of purifying the above-described outer membrane meningococcal group B porin protein or fusion protein comprising: lysing the transformed *E. coli* to release the meningococcal group B porin protein or fusion protein as part of insoluble inclusion bodies; washing the inclusion bodies with a buffer to remove

contaminating *E. coli* cellular proteins; resuspending and dissolving the inclusion bodies in an aqueous solution of a denaturant; diluting the resultant solution in a detergent; and purifying the solubilized meningococcal group B porin protein by gel filtration.

The lysing step may be carried out according to any method known to those of ordinary skill in the art, e.g. by sonication, enzyme digestion, osmotic shock, or by passing through a mull press.

The inclusion bodies may be washed with any buffer which is capable of solubilizing the *E. coli* cellular proteins without solubilizing the inclusion bodies comprising the meningococcal group B porin protein. Such buffers include but are not limited to TEN buffer (50 mM Tris HCl, 1 mM EDTA, 100 mM NaCl, pH 8.0), Tricine, Bicine and HEPES.

Denaturants which may be used in the practice of the invention include 2 to 8 M urea or about 2 to 6 M guanidine HCl, more preferably, 4 to 8 M urea or about 4 to 6 M guanidine HCl, and most preferably, about 8 M urea or about 6 M guanidine HCl.

Examples of detergents which can be used to dilute the solubilized meningococcal group B porin protein include, but are not limited to, ionic detergents such as SDS and cetavlon (Calbiochem); non-ionic detergents such as Tween, Triton X, Brij 35 and octyl glucoside; and zwitterionic detergents such as 3,14-Zwittergent, empigen BB and Champs.

Finally, the solubilized outer membrane meningococcal group B porin protein may be purified by gel filtration to separate the high and low molecular weight materials. Types of filtration gels include but are not limited to Sephacryl-300, Sepharose CL-6B, and Bio-Gel A-1.5m. The column is eluted with the buffer used to dilute the solubilized protein. The fractions containing the porin or fusion thereof may then be identified by gel electrophoresis, the fractions pooled, dialyzed, and concentrated.

Finally, substantially pure (>95%) porin protein and fusion protein may be obtained by passing the concentrated fractions through a Q sepharose high performance column.

In another embodiment, the present invention relates to expression of the meningococcal group B porin protein gene which is part of a vector which comprises the T7 promoter, which is inducible. If a promoter is an inducible promoter, then the rate of transcription increases in response to an inducing agent. The T7 promoter is inducible by the addition of isopropyl β-D-thiogalactopyranoside (IPTG) to the culture medium. Alternatively, the Tac promotor or heat shock promotor may be employed. Preferably, the meningococcal group B porin protein gene is expressed from the pET-17 expression vector or the pET-11a expression vector, both of which contain the T7 promoter.

The cloning of the meningococcal group B porin protein gene or fusion gene into an expression vector may be carried out in accordance with conventional techniques, including blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide appropriate termini, filling in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and ligation with appropriate ligases. Reference is made to Sambrook *et al., Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor, New York, Cold Spring Harbor Laboratory Press (1989), for general methods of cloning.

The meningococcal group B porin protein and fusion protein expressed according to the present invention must be properly refolded in order to achieve a structure which is immunologically characteristic of the native protein. In yet another embodiment, the present invention relates to a method of refolding the above-described outer membrane protein and fusion protein comprising: lysing the transformed cells to release the meningococcal group B porin protein or fusion protein as part of insoluble inclusion bodies; washing the inclusion bodies with a buffer to remove contaminating cellular proteins; resuspending and

dissolving the inclusion bodies in an aqueous solution of a denaturant; diluting the resultant solution in a detergent; and purifying the solubilized meningococcal group B porin protein or fusion protein by gel filtration to give the refolded protein in the eluant. Surprisingly, it has been discovered that the folded trimeric meningococcal group B class 2 and class 3 porin proteins and fusion proteins are obtained directly in the eluant from the gel filtration column.

In another preferred embodiment, the present invention relates to a substantially pure refolded outer membrane meningococcal group B porin protein and fusion protein produced according to the above-described methods. A substantially pure protein is a protein that is generally lacking in other cellular *Neisseria meningitidis* components as evidenced by, for example, electrophoresis. Such substantially pure proteins have a purity of >95%, as measured by densitometry on an electrophoretic gel after staining with Coomassie blue or silver stains.

The following examples are illustrative, but not limiting, of the method and compositions of the present invention. Other suitable modifications and adaptations of the variety of conditions and parameters normally encountered in this art which are obvious to those skilled in the art are within the spirit and scope of the present invention.

Examples

Example 1. Cloning of the Class 3 Porin Protein from Group B Neisseria meningitidis

Materials and Methods

Organisms: The Group B *Neisseria meningitidis* strain 8765 (B:15:P1,3) was obtained from Dr. Wendell Zollinger (Walter Reed Army Institute for Research) and grown on agar media previously described (Swanson, J.L., *Infect.*

Immun. 21:292-302 (1978)) in a candle extinction jar in an incubator maintained at 30°C. *Escherichia coli* strains DME558 (from the collection of S. Benson; Silhavy, T.J. *et al.*, "Experiments with Gene Fusions," Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1984). BRE51 (Bremer, E. *et al.*, *FEMS Microbiol. Lett. 33*:173-178 (1986)) and BL21(DE3) were grown on LB agar plates at 37°C.

P1 Transduction: A $P1_{vir}$ lysate of *E. coli* strain DME558 was used to transduce a tetracycline resistance marker to strain BRE51 (Bremer, E., *et al.*, *FEMS Microbiol. Lett. 33*:173-178 (1986)) in which the entire *ompA* gene had been deleted (Silhavy, T.J., *et al.*, *Experiments with Gene Fusions*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1984)). Strain DME558, containing the tetracycline resistance marker in close proximity of the *ompA* gene, was grown in LB medium until it reached a density of approximately 0.6 OD at 600 nm. One tenth of a milliliter of 0.5 M $CaCl_2$ was added to the 10 ml culture and 0.1 ml of a solution containing 1 x 10^9 PFU of $P1_{vir}$. The culture was incubated for 3 hours at 37°C. After this time, the bacterial cell density was visibly reduced. 0.5 ml of chloroform was added and the phage culture stored at 4°C. Because typically 1-2% of the *E. coli* chromosome can be packaged in each phage, the number of phage generated covers the entire bacterial host chromosome, including the tetracycline resistance marker close to the *ompA* gene.

Next, strain BRE51, which lacks the *ompA* gene, was grown in LB medium overnight at 37°C. The overnight culture was diluted 1:50 into fresh LB and grown for 2 hr. The cells were removed by centrifugation and resuspended in MC salts. 0.1 ml of the bacterial cells were mixed with 0.05 of the phage lysate described above and incubated for 20 min. at room temperature. Thereafter, an equal volume of 1 M sodium citrate was added and the bacterial cells were plated out onto LB plates containing 12.5 µg/ml of tetracycline. The plates were incubated overnight at 37°C. Tetracycline resistant (12 µg/ml) transductants were screened for lack of OmpA protein expression by SDS-PAGE

and Western Blot analysis, as described below. The bacteria resistant to the antibiotic have the tetracycline resistance gene integrated into the chromosome very near where the *ompA* gene had been deleted from this strain. One particular strain was designated BRE-T^R.

A second round of phage production was then carried out with the strain BRE-T^R, using the same method as described above. Representatives of this phage population contain both the tetracycline resistance gene and the OmpA deletion. These phage were then collected and stored. These phage were then used to infect *E. coli* BL21(DE3). After infection, the bacteria contain the tetracycline resistance marker. In addition, there is a high probability that the OmpA deletion was selected on the LB plates containing tetracycline.

Colonies of bacteria which grew on the plates were grown up separately in LB medium and tested for the presence of the OmpA protein. Of those colonies selected for examination, all lacked the OmpA protein as judged by antibody reactivity on SDS-PAGE western blots.

SDS-PAGE and Western Blot: The SDS-PAGE was a variation of Laemmli's method (Laemmli, U.K., *Nature 227*:680-685 (1970)) as described previously (Blake and Gotschlich, *J. Exp. Med. 159*:452-462 (1984)). Electrophoretic transfer to Immobilon P (Millipore Corp. Bedford, MA) was performed according to the methods of Towbin *et al.* (Towbin, H., *et al.*, *Proc. Natl. Acad. Sci. USA 76*:4350-4354 (1979)) with the exception that the paper was first wetted in methanol. The Western blots were probed with phosphatase conjugated reagents (Blake, M.S., *et al.*, *Analyt. Biochem. 136*:175-179 (1984)).

Polymerase Chain Reaction: The method described by Feavers *et al.* (Feavers, I.M., *et al.*, *Infect. Immun. 60*:3620-3629 (1992)) was used to amplify the gene encoding the PorB. The primers selected were primers 33 (GGG GTA GAT CTG CAG GTT ACC TTG TAC GGT ACA ATT AAA GCA GGC GT) and 34 (GGG GGG GTG ACC CTC GAG TTA GAA TTT GTG ACG CAG ACC AAC) as previously described (Feavers, I.M., *et al.*, *Infect. Immun. 60*:3620-3629 (1992)). Briefly, the reaction components were as follows:

Meningococcal strain 8765 chromosomal DNA (100 ng/μl), 1 μl; 5′ and 3′ primers (1 μM) 2 μl each; dNTP (10 mM stocks), 4 μl each; 10 X PCR reaction buffer (100 mM Tris HCl, 500 mM KCl, pH 8.3), 10 μl; 25 mM $MgCl_2$, 6 μl; double distilled H_20, 62 μl; and Taq polymerase (Cetus Corp., 5 u/μl), 1 μl. The reaction was carried out in a GTC-2 Genetic Thermocycler (Precision Inst. Inc, Chicago, IL) connected to a Lauda 4/K methanol/water cooling system (Brinkman Instruments, Inc., Westbury, NY) set at 0°C. The thermocycler was programmed to cycle 30 times through: 94°C, 2 min.; 40°C, 2 min.; and 72°C, 3 min. At the end of these 30 cycles, the reaction was extended at 72°C for 3 min and finally held at 4°C until readied for analysis on a 1% agarose gel in TAE buffer as described by Maniatis (Maniatis, T., *et al.*, *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982)).

Subcloning of the PCR product: The pET-17b plasmid (Novagen, Inc.) was used for subcloning and was prepared by double digesting the plasmid with the restriction endonucleases *Bam*HI and *Xho*I (New England Biolabs, Inc., Beverly, MA). The digested ends were then dephosphorylated with calf intestinal alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN). The digested plasmid was then analyzed on a 1% agarose gel, the cut plasmid removed, and purified using the GeneClean kit (Bio101, La Jolla, CA). The PCR product was prepared by extraction with phenol-chloroform, chloroform, and finally purified using the GeneClean Kit (Bio101). The PCR product was digested with restriction endonucleases *Bgl*II and *Xho*I (New England Biolabs, Inc.). The DNA was then extracted with phenol-chloroform, precipitated by adding 0.1 volumes of 3 M sodium acetate, 5 μl glycogen (20 μg/μl), and 2.5 volumes of ethanol. After washing the DNA with 70% ethanol (vol/vol), it was redissolved in TE buffer. The digested PCR product was ligated to the double digested pET-17b plasmid described above using the standard T4 ligase procedure at 16°C overnight (*Current Protocols in Molecular Biology*, John Wiley & Sons, New York (1993)). The ligation product was then transformed into the BL21 (DE3)-

Δ*omp*A described above which were made competent by the method of Chung *et al.* (Chung, C.T., *et al.*, *Proc. Natl. Acad. Sci. USA 86*:2172-2175 (1989)). The transformants were selected on LB plates containing 50 μg/ml carbenicillin and 12μg/ml tetracycline. Several transformants were selected, cultured in LB both containing carbenicillin and tetracycline for 6 hours at 30°C, and plasmid gene expression inducted by the addition of IPTG. The temperature was raised to 37°C and the cultures continued for an additional 2 hrs. The cells of each culture were collected by centrifugation, whole cell lysates prepared, and analyzed by SDS-PAGE and Western Blot using a monoclonal antibody (4D11) which reacts with all neisserial porins.

Nucleotide Sequence Analysis: The nucleotide sequences of the cloned Class 3 porin gene DNA were determined by the dideoxy method using denatured double-stranded plasmid DNA as the template as described (*Current Protocols in Molecular Biology*, John Wiley & Sons, New York (1993)). Sequenase II kits (United States Biochemical Corp., Cleveland, OH) were used in accordance with the manufacturer's instructions. The three synthesized oligonucleotide primers (Operon Technologies, Inc., Alameda, CA) were used for these reactions. One for the 5′ end, which consisted of 5′TCAAGCTTGGTACCGAGCTC and two for the 3′ end, 5′TTTGTTAGCAGCCGGATCTG and 5′ CTCAAGACCCGTTTAGAGGCC. Overlapping, nested deletions were made by linearizing the plasmid DNA by restriction endonuclease *Bpu*1102I and the ends blunted by the addition of Thio-dNTP and Klenow polymerase (*Current Protocols in Molecular Biology*, John Wiley & Sons, New York (1993)). The linearized plasmid was then cleaved with restriction endonuclease *Xho*I and the exoII/Mung bean nuclease deletion kit used to make 3′ deletions of the plasmid (Stratagene, Inc., La Jolla, CA) as instructed by the supplier. A map of this strategy is shown in Figure 1.

Expression and purification of the PorB gene product: Using a sterile micropipette tip, a single colony of the BL21 (DE3)-Δ*omp*A containing the PorB-pET-17b plasmid was selected and inoculated into 10 ml of LB broth containing

50 µg/ml carbenicillin. The culture was incubated overnight at 30°C while shaking. The 10 ml overnight culture was then sterilely added to 1 liter of LB broth with the same concentration of carbenicillin, and the culture continued in a shaking incubator at 37°C until the OD_{600} reached 0.6-1.0. Three mls of a stock solution of IPTG (100 mM) was added to the culture and the culture incubated for an additional 30 min. Rifampicin was then added (5.88 ml of a stock solution; 34 mg/ml in methanol) and the culture continued for an additional 2 hrs. The cells were harvested by centrifugation at 10,000 rpm in a GS3 rotor for 10 min and weighed. The cells were thoroughly resuspended in 3 ml of TEN buffer (50 mM Tris HCl, 1 mM Tris HCl, 1 mM EDTA, 100 mM NaCl, pH 8.0) per gram wet weight of cells. To this was added 8 µl of PMSF stock solution (50 mM in anhydrous ethanol) and 80 µl of a lysozyme stock solution (10 mg/ml in water) per gram wet weight of cells. This mixture was stirred at room temperature for 20 min. While stirring, 4 mg per gram wet weight of cells of deoxycholate was added. The mixture was placed in a 37°C water bath and stirred with a glass rod. When the mixture became viscous, 20 µl of DNase I stock solution (1 mg/ml) was added per gram weight wet cells. The mixture was then removed from the water bath and left at room temperature until the solution was no longer viscous. The mixture was then centrifuged at 15,000 rpm in a SS-34 rotor for 20 min at 4°C. The pellet was retained and thoroughly washed twice with TEN buffer. The pellet was then resuspended in freshly prepared TEN buffer containing 0.1 mM PMSF and 8 M urea and sonicated in a bath sonicator (Heat Systems, Inc., Plain view, NY). The protein concentration was determined using a BCA kit (Pierce, Rockville, IL) and the protein concentration adjusted to less than 10 mg/ml using the TEN-urea buffer. The sample was then diluted 1:1 with 10% (weight/vol) Zwittergent 3,14 (Calbiochem, La Jolla, CA), sonicated, and loaded onto a Sephacryl S-300 molecular sieve column. The Sephacryl S-300 column (2.5 cm x 200 cm) had previously equilibrated with 100 mM Tris HCl, 200 mM NaCl, 10 mM EDTA, 0.05% Zwittergent 3,14, and 0.02% azide, pH 8.0. The column flow rate was adjusted to 8 ml/hr and 10 ml fractions were collected. The

OD_{280} of each fraction was measured and SDS-PAGE analysis performed on protein containing fractions.

Inhibition ELISA Assays: Microtiter plates (Nunc-Immuno Plate IIF, Nunc, Inc., Naperville, IL) were sensitized by adding 0.1 ml per well of porB (2 µg/ml) purified from the wild type strain 8765, in 0.1 M carbonate buffer, pH 9.6 with 0.02% azide. The plates were incubated overnight at room temperature. The plates were washed five times with 0.9% NaCl, 0.05% Brij 35, 10 mM sodium acetate pH 7.0, 0.02% azide. Human immune sera raised against the Type 15 Class 3 PorB protein was obtained from Dr. Phillip O. Livingston, Memorial-Sloan Kettering Cancer Center, New York, N.Y. The human immune sera was diluted in PBS with 0.5% Brij 35 and added to the plate and incubated for 2 hr at room temperature. The plates were again washed as before and the secondary antibody, alkaline phosphatase conjugated goat anti-human IgG (Tago Inc., Burlingame, CA), was diluted in PBS-Brij, added to the plates and incubated for 1 hr at room temperature. The plates were washed as before and *p*-nitrophenyl phosphate (Sigma Phosphatase Substrate 104) (1 mg/ml) in 0.1 diethanolamine, 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 0.02% azide, pH 9.8, was added. The plates were incubated at 37°C for 1 h and the absorbance at 405 nm determined using an Elida-5 microtiter plate reader (Physica, New York, NY). Control wells lacked either the primary and/or secondary antibody. This was done to obtain a titer for each human serum which would give a half-maximal reading in the ELISA assay. This titer for each human serum would be used in the inhibition ELISA. The ELISA microtiter plate would be sensitized with purified wild type PorB protein and washed as before. In a separate V-96 polypropylene microtiter plate (Nunc, Inc.), varying amounts of either purified wild type PorB protein or the purified recombinant PorB protein were added in a total volume of 75 µl. The human sera were diluted in PBS-Brij solution to twice their half maximal titer and 75 µl added to each of the wells containing the PorB or recombinant PorB proteins. This plate was incubated for 2 hr at room temperature and centrifuged in a Sorvall RT6000 refrigerated centrifuge,

equipped with microtiter plate carriers (Wilmington, DE) at 3000 rpm for 10 min. Avoiding the V-bottom, 100 µl from each well was removed and transferred to the sensitized and washed ELISA microtiter plate. The ELISA plates are incubated for an additional 2 hr, washed, and the conjugated second antibody added as before. The plate is then processed and read as described. The percentage of inhibition is then processed and read as described. The percentage of inhibition is calculated as follows:

$$\frac{1 - (ELISA\ value\ with\ either\ PorB\ or\ rPorB\ protein\ added)}{(ELISA\ value\ without\ the\ porB\ added)} \times 100$$

Results

Polymerase Chain Reaction and Subcloning: A method to easily clone, genetically manipulate, and eventually obtain enough pure porin protein from any number of different neisserial porin genes for further antigenic and biophysical characterization has been developed. The first step toward this goal was cloning the porin gene from a Neisseria. Using a technique originally described by Feavers, *et al.* (Feavers, I.M., *et al.*, *Infect. Immun. 60*:3620-3629 (1992)), the DNA sequence of the mature porin protein from a class 3, serotype 15 porin was amplified using the chromosome of meningococcal strain 8765 as a template for the PCR reaction. Appropriate endonuclease restriction sites had been synthesized onto the ends of the oligonucleotide primers, such that when cleaved, the amplified mature porin sequence could be directly ligated and cloned into the chosen expression plasmid. After 30 cycles, the PCR products shown in Figure 2 were obtained. The major product migrated between 900bp and 1000bp which was in accord with the previous study (Feavers, I.M., *et al.*, *Infect. Immun. 60*:3620-3629 (1992)). However, a higher molecular weight product was not

seen, even though the PCR was conducted under low annealing stringencies (40°C; 50 mM KCl).

To be able to produce large amounts of the cloned porin protein, the tightly controlled expression system of Studier, *et al.* (Studier and Moffatt, *J. Mol. Biol. 189*:113-130 (1986)) was employed, which is commercially available through Novagen Inc. The amplified PCR product was cloned into the *Bam*HI-*Xho*I site of plasmid pET-17b. This strategy places the DNA sequence for the mature porin protein in frame directly behind the T7 promoter, the DNA sequence encoding for the 9 amino acid leader sequence and 11 amino acids of the mature ϕ10 protein. The Studier *E. coli* strain BL21 lysogenic for the DE3 lambda derivative (Studier and Moffatt, *J. Mol. Biol. 189*:113-130 (1986)) was selected as the expression host for the pET-17b plasmid containing the porin gene. But because it was thought that the OmpA protein, originating from the *E. coli* expression host, might tend to co-purify with the expressed meningococcal porin protein, a modification of this strain was made by P1 transduction which eliminated the *ompA* gene from this strain. Thus, after restriction endonuclease digestion of both the PCR product and the pET-17b vector and ligation, the product was transformed into BL21(DE3)-Δ*ompA* and transformants selected for ampicillin and tetracycline resistance. The restriction map of pET-17b is shown in Figure 11A, while the nucleotide sequence between the *Bgl*II and *Xho*I sites of pET-17b is shown in Figure 11B. Of the numerous colonies observed on the selection plate, 10 were picked for further characterization. All ten expressed large amounts of a protein, which migrated at the approximate molecular weight of the PorB protein, when grown to log phase and induced with IPTG. The whole cell lysate of one such culture is shown in Figure 3a. The western blot analysis with the 4D11 monoclonal antibody further suggested that the protein being expressed was the PorB protein (Figure 3b). As opposed to other studies, when neisserial porins have been cloned and expressed in *E. coli*, the host bacterial cells showed no signs of any toxic or lethal effects even after the addition of the IPTG.

The *E. coli* cells appeared viable and could be recultured at any time throughout the expression phase.

Nucleotide sequence analysis: The amount of PorB expressed in these experiments was significantly greater than that previously observed and there appeared to be no adverse effects of this expression on the host *E. coli*. To be certain that no PCR artifacts had been introduced into the meningococcal porin gene to allow for such high expression, the entire ϕ10 porin fusion was sequenced by double stranded primer extension from the plasmid. The results are shown in Figure 4. The nucleotide sequence was identical with another meningococcal serotype 15 *Por*B gene sequence previously reported by Heckels, *et al.* (Ward, M.J., *et al.*, *FEMS Microbiol. Lett. 73*:283-289 (1992)) with two exceptions which are shown. These two nucleotide differences each occur in the third position of the codon and would not alter the amino acid sequence of the expressed protein. Thus, from the nucleotide sequence, there did not appear to be any PCR artifact or mutation which might account for the high protein expression and lack of toxicity within the *E. coli*. Furthermore, this data would suggest that a true PorB protein was being produced.

Purification of the expressed porB gene product: The PorB protein expressed in the *E. coli* was insoluble in TEN buffer which suggested that when expressed, the PorB protein formed into inclusion bodies. However, washing of the insoluble PorB protein with TEN buffer removed most of the contaminating *E. coli* proteins. The PorB protein could then be solubilized in freshly prepared 8M urea and diluted into the Zwittergent 3,14 detergent. The final purification was accomplished, using a Sephacryl S-300 molecular sieve column which not only removed the urea but also the remaining contaminating proteins. The majority of the PorB protein eluted from the column having the apparent molecular weight of trimers much like the wild type PorB. The comparative elution patterns of both the wild type and the PorB expressed in the *E. coli* are shown in Figure 5. It is important to note that when the PorB protein concentration in the 8 M urea was in excess of 10 mg/ml prior to dilution into the

Zwittergent detergent, the relative amounts of PorB protein found as trimers decreased and appeared as aggregates eluting at the void volume. However, at protein concentrations below 10 mg/ml in the urea buffer, the majority of the PorB eluted in the exact same fraction as did the wild type PorB. It was also determined using a T7-Tag monoclonal antibody and western blot analysis that the 11 amino acids of the mature T7 capsid protein were retained as the amino terminus. The total yield of the meningococcal porin protein from one liter of *E. coli* was approximately 50 mg.

Inhibition ELISA Assays. In order to determine if the purified trimeric recombinant PorB had a similar antigenic conformation as compared to the PorB produced in the wild type meningococcal strain 8765, the sera from six patients which had been vaccinated with the wild type meningococcal Type 15 PorB protein were used in inhibition ELISA assays. In the inhibition assay, antibodies reactive to the native PorB were competitively inhibited with various amounts of either the purified recombinant PorB or the homologous purified wild type PorB. The results of the inhibition with the homologous purified PorB of each of the six human sera and the mean inhibition of these sera are shown in Figure 6. The corresponding inhibition of these sera with the purified recombinant PorB is seen in Figure 6B. A comparison of the mean inhibition from Figure 6 and 7 are plotted in Figure 8. These data would suggest that the antibodies contained in the sera of these six patients found similar epitopes on both the homologous purified wild type PorB and the purified recombinant PorB. This gave further evidence that the recombinant PorB had regained most if not all of the native conformation found in the wild type PorB.

Example 2. Cloning of the Class 2 Porin from Group B Neisseria Meningitidis strain BNCV M986

Genomic DNA was isolated from approximately 0.5g of Group B *Neisseria meningitidis* strain BNCV M986 (serotype 2a) using previously described methods (Sambrook *et al., Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor, New York, Cold Spring Harbor Laboratory Press (1989)). This DNA then served as the template for two class 2 porin specific oligonucleotides in a standard PCR reaction. These oligonucleotides were designed to be complementary to the 5′ and 3′ flanking regions of the class 2 porin and to contain *Eco*RI restriction sites to facilitate the cloning of the fragment. The sequences of the oligonucleotides were as follows:

5′ AGC GGC TTG *GAA TTC* CCG GCT GGC TTA AAT TTC 3′ and

5′ CAA ACG AAT *GAA TTC* AAA TAA AAA AGC CTG 3′.

The polymerase chain reaction was then utilized to obtain the class 2 porin. The reaction conditions were as follows: BNCV M986 genomic DNA 200ng, the two oligonucleotide primers described above at 1 μM of each, 200 μM of each dNTP, PCR reaction buffer (10 mM Tris HCl, 50 mM KCl, pH 8.3), 1.5 mM $MgCl_2$, and 2.5 units of *Taq* polymerase, made up to 100 μl with distilled H_2O. This reaction mixture was then subjected to 25 cycles of 95°C for 1 min, 50°C for 2 min and 72°C for 1.5 min. At the end of the cycling period, the reaction mixture was loaded on a 1% agarose gel and the material was electrophoresed for 2h after which the band at 1.3 kb was removed and the DNA recovered using the Gene Clean kit (Bio 101). This DNA was then digested with *Eco*RI, repurified and ligated to *Eco*RI digested pUC19 using T_4 DNA ligase. The ligation mixture was used to transform competent *E. coli* DH5α. Recombinant plasmids were selected and sequenced. The insert was found to have a DNA sequence consistent with that of a class 2 porin. *See*, Murakami, K. *et al., Infect. Immun.* 57:2318-2323 (1989).

The plasmid pET-17b (Novagen) was used to express the class 2 porin. As described below, two plasmids were constructed that yielded two different proteins. One plasmid was designed to produce a mature class 2 porin while the other was designed to yield a class 2 porin fused to 20 amino acids from the T7 gene ϕ10 capsid protein.

Construction of the mature class 2 porin

The mature class 2 porin was constructed by amplifying the pUC19-class 2 porin construct using the oligonucleotides: 5'-CCT GTT GCA GCA *CAT ATG* GAC GTT ACC TTG TAC GGT ACA ATT AAA GC-3' and 5 '-CGA CAG GCT TTT TCT CGA GAC CAA TCT TTT CAG -3'. This strategy allowed the cloning of the amplified class 2 porin into the *Nde*I and *Xho*I sites of the plasmid pET-17b thus producing a mature class 2 porin. Standard PCR was conducted using the pUC19-class 2 as the template and the two oligonucleotides described above. This PCR reaction yielded a 1.1kb product when analyzed on a 1.0% agarose gel. The DNA obtained from the PCR reaction was gel purified and digested with the restriction enzymes *Nde*I and *Xho*I. The 1.1kb DNA produced was again gel purified and ligated to *Nde*I and *Xho*I digested pET-17b using T_4 DNA ligase. This ligation mixture was then used to transform competent *E. coli* DH5α. Colonies that contained the 1.1kb insert were chosen for further analysis. The DNA from the DH5α clones was analyzed by restriction mapping and the cloning junctions of the chosen plasmids were sequenced. After this analysis, the DNA obtained from the DH5α clones was used to transform *E. coli* BL21(DE3)-Δ*ompA*. The transformants were selected to LB-agar containing 100 μg/ml of carbenicillin. Several transformants were screened for their ability to make the class 2 porin protein. This was done by growing the clones in LB liquid medium containing 100 μg/ml of carbenicillin and 0.4% glucose at 30°C to OD_{600} = 0.6 then inducing the cultures with IPTG (0.4 mM). The cells were then disrupted and the cell extract was analyzed by SDS-PAGE. The nucleotide sequence and

translated amino acid sequence of the mature class II porin gene cloned into pET-17b are shown in Figures 9A and 9B.

Construction of the fusion class 2 porin

The fusion class 2 porin was constructed by amplifying the pUC19-class 2 porin construct using the oligonucleotides: 5'-CCT GTT GCA GCG GAT CCA GAC GTT ACC TTG TAC GGT ACA ATT AAA GC- 3' and 5'-CGA CAG GCT TTT TCT CGA GAC CAA TCT TTT CAG -3'. This strategy allowed the cloning of the amplified class 2 porin into the *Bam*HI and *Xho*I sites of the plasmid pET-17b thus producing a fusion class 2 porin containing an additional 22 amino acids at the N-terminus derived from the T7 ϕ10 capsid protein contained in the plasmid. Standard PCR was conducted using the pUC19-class 2 as the template and the two oligonucleotides described above. The PCR reaction yielded a 1.1kb product when analyzed on a 1.0% agarose gel. The DNA obtained from the PCR reaction was gel purified and digested with the reaction enzymes *Bam*HI and *Xho*I. The 1.1kb product produced was again gel purified and ligated to *Bam*HI and *Xho*I digested pET-17b using T_4 DNA ligase. This ligation mixture was then used to transform competent *E. coli* DH5α. Colonies that contained the 1.1kb insert were chosen for further analysis. The DNA from the DH5α clones was analyzed by restriction enzyme mapping and the cloning junctions of the chosen plasmids were sequenced. The nucleotide sequence and translated amino acid sequence of the fusion class II porin gene cloned into the expression plasmid pET-17b are shown in Figures 10A and 10B. After this analysis, the DNA obtained from the DH5α clones was used to transform *E. coli* BL21(DE3)-Δ*ompA*. The transformants were selected on LB-agar containing 100 μg/ml of carbenicillin. Several transformants were screened for their ability to make the class 2 porin protein. This was done by growing the clones in LB liquid medium containing 100 μg/ml of carbenicillin and 0.4% glucose at 30°C to OD_{600}

= 0.6 then inducing the cultures with IPTG (0.4 mM). The cells were then disrupted and the cell extract was analyzed by SDS-PAGE.

Example 3. Cloning and Expression of the Mature class 3 porin from Group B Neisseria meningitidis strain 8765 in E. coli

Genomic DNA was isolated from approximately 0.5 g of Group B *Neisseria meningitidis* strain 8765 using the method described above (Sambrook *et al., Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor, New York, Cold Spring Harbor Laboratory Press (1989)). This DNA then served as the template for two class 3 porin specific oligonucleotides in a standard PCR reaction.

The mature class 3 porin was constructed by amplifying the genomic DNA from 8765 using the oligonucleotides: 5'-GTT GCA GCA *CAT ATG* GAC GTT ACC CTG TAC GGC ACC-3' and 5'-GGG GGG ATG GAT CCA GAT TAG AAT TTG TGG CGC AGA CCG ACA CC-3'. This strategy allowed the cloning of the amplified class 3 porin into the *Nde*I and *BamH*I sites of the plasmid pET-24a+ (Figures 13A and 13B), thus producing a mature class 3 porin. Standard PCR was conducted using the genomic DNA isolated from 8765 as the template and the two oligonucleotides described above.

The reaction conditions were as follows: 8765 genomic DNA 200 ng, the two oligonucleotide primers described above at 1 μM of each, 200 μM of each dNTP, PCR reaction buffer (10 mM Tris HCl, 50 mM KCl, pH 8.3), 1.5 mM $MgCl_2$, and 2.5 units of *Taq* polymerase, and made up to 100 μl with distilled water. This reaction mixture was then subjected to 25 cycles of 95°C for 1 min, 50°C for 2 min and 72°C for 1.5 min.

This PCR reaction yielded about 930 bp of product, as analyzed on a 1% agarose gel. The DNA obtained from the PCR reaction was gel purified and digested with the restriction enzymes *Nde*I and *Bam*HI. The 930 bp product was

again gel purified and ligated to *Nde*I and *Bam*HI digested pET-24a(+) using T4 ligase. This ligation mixture was then used to transform competent *E. coli* DH5α. Colonies that contained the 930 bp insert were chosen for further analysis. The DNA from the *E. coli* DH5α clones was analyzed by restriction enzyme mapping and cloning junctions of the chosen plasmids were sequenced. After this analysis, the DNA obtained from the *E. coli* DH5α clones was used to transform *E. coli* BL21(DE3)-Δ*ompA*. The transformants were selected on LB-agar containing 50 μg/ml of kanamycin. Several transformants were screened for their ability to make the class 3 porin protein. This was done by growing the clones in LB liquid medium containing 50 μg/ml of kanamycin and 0.4% of glucose at 30°C to OD_{600} = 0.6 then inducing the cultures with IPTG (1 mM). The cells were then disrupted and the cell extract was analyzed by SDS-PAGE.

Example 4. Purification and refolding of recombinant class 2 porin

E coli strain BL21(DE3)Δ*omp*A [pNV-5] is grown to mid-log phase (OD = 0.6 at 600 nm) in Luria broth at 30°C. IPTG is then added (0.4 mM final) and the cells grown an additional two hours at 37°C. The cells were then harvested and washed with several volumes of TEN buffer (50 mM Tris-HCl, 0.2 M NaCl, 10 mM EDTA, pH = 8.0) and the cell paste stored frozen at -75°C.

For purification preweighed cells are thawed and suspended in TEN buffer at a 1:15 ratio (g/v). The suspension is passed through a Stansted cell disrupter (Stansted fluid power Ltd.) twice at 8.000 psi. The resultant solution is then centrifuged at 13,000 rpm for 20 min and the supernatant discarded. The pellet is then twice suspended in TEN buffer containing 0.5% deoxycholate and the supernatants discarded. The pellet is then suspended in TEN buffer containing 8 M deionized urea (electrophoresis grade) and 0.1 mM PMSF (3 g/10ml). The suspension is sonicated for 10 min or until an even suspension is achieved. 10 ml of a 10% aqueous solution of 3,14-zwittergen (Calbiochem) is

added and the solution thoroughly mixed. The solution is again sonicated for 10 min. Any residual insoluble material is removed by centrifugation. The protein concentration is determined and the protein concentration adjusted to 2 mg/ml with 8 M urea-10% zwittergen buffer (1:1 ratio).

This mixture is then applied to a 2.6 x 100 cm column of Sephacryl S-300 equilibrated in 100 mM Tris-HCl, 1 M NaCl, 10 mM EDTA, 20 mM $CaCl_2$, 0.05% 3,14-zwittergen, 0.02% sodium azide, pH = 8.0. The flow rate is maintained at 1 ml/min. Fractions of 10 ml are collected. The porin refolds into trimer during the gel filtration. The OD = 280 nm of each fraction is measured and those fractions containing protein are subjected to SDS gel electrophoresis assay for porin. Those fractions containing porin are pooled. The pooled fractions are either dialyzed or diluted 1:10 in 50 mM Tris HCl pH = 8.0, 0.05% 3,14-zwittergen, 5 mM EDTA, 0.1 M NaCl. The resulting solution is then applied to a 2.6 x 10 cm Q sepharose high performance column (Pharmacia) equilibrated in the same buffer. The porin is eluted with a linear gradient of 0.1 to 1 M NaCl.

Example 5. Purification and refolding of recombinant class 3 porin

E coli strain BL21 (DE3) Δ*omp*A containing the porB-pET-17b plasmid is grown to mid-log phase (OD = 0.6 at 600 nm) in Luria broth at 30°C. IPTG is then added (0.4 mM final) and the cells grown an additional two hours at 37°C. The cells were then harvested and washed with several volumes of TEN buffer (50 mM Tris-HCl, 0.2 M NaCl, 10 mM EDTA, pH = 8.0) and the cell paste stored frozen at -75°C.

For purification about 3 grams of cells are thawed and suspended in 9 ml of TEN buffer. Lysozyme is added (Sigma, 0.25 mg/ml) deoxycholate (Sigma, 1.3 mg/ml) plus PMSF (Sigma, μg/ml) and the mixture gently shaken for one hour at room temperature. During this time, the cells lyse and the released DNA

causes the solution to become very viscous. DNase is then added (Sigma, 2 μg/ml) and the solution again mixed for one hour at room temperature. The mixture is then centrifuged at 15K rpm in a S-600 rotor for 30 minutes and the supernatant discarded. The pellet is then twice suspended in 10 ml of TEN buffer and the supernatants discarded. The pellet is then suspended in 10 ml of 8 M urea (Pierce) in TEN buffer. The mixture is gently stirred to break up any clumps. The suspension is sonicated for 20 minutes or until an even suspension is achieved. 10 ml of a 10% aqueous solution of 3,14-zwittergen (Calbiochem) is added and the solution thoroughly mixed. The solution is again sonicated for 10 minutes. Any residual insoluble material is removed by centrifugation. The protein concentration is determined and the protein concentration adjusted to 2 mg/ml with 8 M urea-10% zwittergen buffer (1:1 ratio).

This mixture is then applied to a 180 x 2.5 cm column of Sephacryl S-300 (Pharmacia) equilibrated in 100 mM Tris-HCl, 1 M NaCl, 10 mM EDTA, 20 mM $CaCl_2$, 0.05% 3,14-zwittergen, pH = 8.0. The flow rate is maintained at 1 ml/min. Fractions of 10 ml are collected. The porin refolds into trimer during the gel filtration. The OD_{280} nm of each fraction is measured and those fractions containing protein are subjected to SDS gel electrophoresis assay for porin. Those fractions containing porin are pooled.

The pooled fractions are dialyzed and concentrated 4-6 fold using Amicon concentrator with a PM 10 membrane against buffer containing 100 mM Tris-HCl, 0.1 M NaCl, 10 mM EDTA, 0.05% 3,14-zwittergen, pH = 8.0. Alternatively, the pooled fractions are precipitated with 80% ethanol and resuspended with the above-mentioned buffer. Six to 10 mg of the material is then applied to a monoQ 10/10 column (Pharmacia) equilibrated in the same buffer. The porin is eluted from a shallow 0.1 to 0.6 M NaCl gradient with a 1.2% increase per min over a 50 min period. The Flow rate is 1 ml/min. The peak containing porin is collected and dialyzed against TEN buffer and 0.05% 3,14-zwittergen. The porin may be purified further by another S-300 chromatography.

Example 6. Purification and chemical modification of the polysaccharides

The capsular polysaccharide from both group B *Neisseria meningitidis* and *E. coli* K1 consists of α(2→8) polysialic acid (commonly referred to as GBMP or K1 polysaccharide). High molecular weight polysaccharide isolated from growth medium by precipitation (*see*, Frasch, C.E., "Production and Control of *Neisseria meningitidis* Vaccines" in *Bacterial Vaccines*, Alan R. Liss, Inc., pages 123-145 (1990)) was purified and chemically modified before being coupled to the porin protein. The high molecular weight polysaccharide was partially depolymerized with 0.1 M acetic acid (7 mg polysaccharide/ml), pH = 6.0 to 6.5 (70°C, 3 hrs) to provide polysaccharide having an average molecular weight of 12,000-16,000. After purification by gel filtration column chromatography (Superdex 200 prep grade, Pharmacia), the polysaccharide was N-deacetylated in the presence of $NaBH_4$ and then N-propionylated as described by Jennings *et al.* (*J. Immunol. 137*:1808 (1986)) to afford N-Pr GBMP (see Example 14). Treatment with $NaIO_4$ followed by gel filtration column purification gave the oxidized N-Pr GBMP having an average molecular weight of 12,000 daltons.

Example 7. Coupling of oxidized N-Pr GBMP to the group B meningococcal class 3 porin protein (PP)

The oxidized N-Pr GBMP (9.5 mg) was added to purified class 3 porin protein (3.4 mg) dissolved in 0.21 ml of 0.2 M phosphate buffer pH 7.5 which also contained 10% octyl glucoside. After the polysaccharide was dissolved, sodium cyanoborohydride (7 mg) was added and the reaction solution was incubated at 37°C for 4 days. The reaction mixture was diluted with 0.15 M sodium chloride solution containing 0.01% thimerosal and separated by gel filtration column chromatography using Superdex 200 PG. The conjugate (N-Pr

GBMP-PP) was obtained as single peak eluting near the void volume. Analysis of the conjugate solution for sialic acid and protein showed that the conjugate consists of 43% polysaccharide by weight. The porin protein was recovered in the conjugate in 44% yield and the polysaccharide in 12% yield. The protein recoveries in different experiments generally occur in the 50-80% range and those of the polysaccharide in the 9-13% range (see also Example 14).

Example 8. Immunogenicity studies

The immunogenicities of the N-Pr GBMP-PP conjugate and those of the N-Pr GBMP-Tetanus toxoid (N-Pr GBMP-TT) conjugate which was prepared by a similar coupling procedure were assayed in 4-6 week old outbread Swiss Webster CFW female mice. The polysaccharide (2 μg)-conjugate was administered on days 1, 14 and 28, and the sera collected on day 38. The conjugates were administered as saline solutions, adsorbed on aluminum hydroxide, or admixed with stearyl tyrosine. The sera ELISA titers against the polysaccharide antigen and bactericidal titers against *N. meningitidis* group B are summarized in Table 1.

Example 9. Expression of group B Neisseria meningitidis Outer Membrane (MB3) Using Yeast Pichia pastoris Expression System

Materials and Methods

Strains and Plasmids

Pichia pastoris GS 115 (provided by Invitrogen) has a defect in the histidinol dehydrogenase gene (his4) which prevents it from synthesizing histidine. All expression plasmids carry the HIS4 gene which complements his4

in the host, so transformants are selected for their ability to grow on histidine-deficient medium. Until transformed, GS 115 will not grow on minimal medium alone.

Expression vectors

Four different expression vectors were used that include the strong, highly-inducible AOX1 promoter for expression of foreign protein (*Pichia* Expression Kit, Invitrogen). One vector, pHIL-D2, is used for intracellular expression, while the other three (pHIL-S1, pPIC9, and pPIC9K) are used for secreted expression. Maps of the pHIL-D2, pHIL-S1, and pPIC9 vectors may be found on pp. 19-22 of the Invitrogen Instruction Manual for the *Pichia* Expression Kit, Version E, the contents of which is hereby incorporated by reference. Secretion requires the presence of a signal sequence on the expressed protein to target it to the secretory pathway. To improve the chances for success, two different kinds of vectors are included in the kit. The vector pHIL-S1 carries a native *Pichia pastoris* signal from the acid phosphatase gene, PHO1. The vectors, pPIC9 and pPIC9K (with corrected HIS4 region), both carry the secretion signal from the *S. cerevisiae* α-mating factor pre-pro peptide. The advantage of expressing secreted proteins is that *P. pastoris* secretes very low levels of native proteins. Thus, the secreted heterologous protein comprises the vast majority of the total protein in the media and serves as the first step in purification of the protein (Barr *et al.*, *Pharm. Eng. 12(2)*:48-51 (1992)).

Cloning of the meningococcal B class 3 protein gene (MB3)

The genomic DNA of Group B *Neisseria meningitidis* (strain 8765) served as the template for the amplification of class 3 porin (MB3) in a standard PCR. The amplified DNA fragment (930 b.p. long) of the mature porin protein was ligated in Nde I - BamH I cloning sites of the pET-24a cloning/expression

vector, originally constructed by Studier *et al.*, *J. Mol. Biol. 189*:113-130 (1986); *Meth. Enzymol. 185*:60-89(1990); *J. Mol. Biol. 219*:37-44 (1991), and manufactured by Novagen. The pET vectors were developed for cloning and for expressing target DNA fragments under the strong T7 transcription and translation signals. Expression from the T7 promoter is induced by providing the host cell with a source of T7 RNA polymerase. Newer, more convenient vectors utilizing the T7 expression system are now available from Novagen (Madison, WI 53711). The T7 expression system was successfully used for the expression of MB3 in *E. coli* (see Example 3).

The optimization of the translation elongation rate for the expressed MB3 gene

Codon usage is known to affect the translational elongation rate, and therefore it has been considered an important factor in affecting product yields (Romanos *et al.*, *Yeast 8*:423-488 (1992)). There is evidence that codon usage may affect both yield and quality of the expressed protein. A number of highly expressed genes show a strong bias toward a subset of codons (Bennetzen *et al.*, *J. Biol. Chem. 257*:3026-3031 (1982). This "major codon bias," which can vary greatly between organisms, is thought to be a growth optimization strategy. This mechanism allows an organism to be capable of efficient translation of highly expressed genes during rapid growth, as only a subset of tRNAs and aminoacyl-tRNA synthetases need to be present in high concentrations. Kurland *et al.*, *TIBS 12*:126-128 (1987). In cases where mRNA contains rare codons, aminoacyl-tRNAs may become limited, increasing the probability of amino acid misincorporations, and possibly causing ribosomes to drop off. Indeed, a high misincorporation frequency has recently been observed in a foreign protein produced in *E. coli* (Scorer *et al.*, *Nucleic Acids Res. 19*:3511-3516 (1991)). Moreover, proteins containing amino acid misincorporations are difficult to purify and may have both impaired activity and antigenicity. The presence of several rare codons has been shown to limit the production of tetanus toxin

fragment C in *E. coli* (Makoff *et al.*, *Nucleic Acids Res. 17*:10191-10201 (1989)). In yeast, Hoekema *et al.* (*Mol. Cell Biol. 7*: 2914-2924 (1987)) showed that substitution of a large proportion of preferred codons for rare codons in the 5′ portion of the PGK (phosphoglycerate kinase) gene caused a decrease in expression levels. Recently, the expression of an immunoglobulin kappa chain in yeast has been shown to be increased 50-fold when a synthetic codon-optimized gene is used, although the level of kappa chain mRNA remains the same.

Significant differences between codon usage profiles of *Pichia* and MB3 were found (Table 5). In order to optimize the translation efficiency, particularly at the beginning of translation elongation, codons optimal for *Pichia* were introduced into the 5′ region of the MB3 gene. When constructing the linker used to clone MB3 into pHIL-S1, the oligomers were synthesized so that they contained sequence optimized for *Pichia* expression. A 51 nucleotide long oligomer (51-mer) was synthesized for this purpose. The sequence of the oligomer is:

5'-TCGAGACGTCACTTTGTACGGTACTATTAAGGCTGGTGTTGAGA CTTCCCG-3'

A 47 nucleotide oligomer complementary to the 51-mer was also synthesized. The sequence of this oligomer is:

5'-CGGGAAGTCTCAACACCAGCCTTAATAGTACCGTACAAAGTGAC GTC-3'

These two oligomers, which contain *Xho*I and *Bsr*I restriction sites, were annealed to serve as a connector, and then ligated to vector pHIL-S1, which had been linearized with *Xho*I digestion. The ligated fragment was then digested with *BamH*I, gel purified, and ligated with an MB3 fragment obtained from cutting the pNV15 vector with both *Bsr*I and *BamH*I enzymes. The fragment was then cloned into the *Pichia* pHIL-S1 expression vector. The new DNA sequence of the 5′ region of MB3 was verified by DNA sequencing of pHIL-S1/MB3 isolated from *Pichia*.

The sequence of the original 5′ end of the gene for mature MB3 (from NT 1) is:

gac gtt acc ctg tac ggc acc att aaa gcc ggc gta gaa act tcc cgc tct gta ttt cac cag aac ggc
D V T L Y G T I K A G V E T S R S V F H Q N G

caa gtt act gaa gtt aca
Q V T E V T

The codon-optimized sequence of the same fragment (replaced nucleotides showed as capital letters), along with its corresponding amino acid sequence is:

gac gtC acT Ttg tac ggT acT att aaG gcT ggT gtT gaG act tcc cgc tct gta ttt cac cag aac
D V T L Y G T I K A G V E T S R S V F H Q N

ggc caa gtt act gaa gtt aca
G Q V T E V T

Vector pHIL-S1/MB3, containing the codon-optimized MB3 DNA, served as the template for the amplification of MB3 in a standard PCR. Oligomers were synthesized to serve as PCR primers. The PCR fragments of MB3 were inserted into *Pichia* expression vectors either directly or by using the Original TA Cloning Kit (Invitrogen); details are given below.

For the cloning of MB3 into the *EcoR*I site of pHIL-D2:

Forward primer (39 nt, having an engineered *EcoR*I site and a sequence (5'ATG) encoding an initiation methionine):

5'-CGAGAATTCATGGACGTCACTTTGTACGGTACTATTAAG-3'

Reverse primer (45 nt, having an engineered *EcoR*I site and stop codon):

5'-GCTGAATTCTTAGAATTTGTGGCGCAGACCGACACCGCCGGCAGT-3'

For the cloning of MB3 into the *EcoR*I-*Avr*II sites of pPIC9 and pPIC9: Forward primer (39 nucleotides (nt), having an engineered *EcoR*I site; no sequence encoding an initiation methionine was necessary because the leader peptide had an initiation methionine):

5'-AGCGAATTCGACGTCACTTTGTACGGTACTATTAAGGCT-3'

Reverse primer (36 nt, having an engineered *Avr*II site and stop codon):

5'-CACCCTAGGTTAGAATTTGTGACGCAGACCGACACC-3'

For PCR amplification of the complete MB3 gene, Vent® DNA polymerase (NEB) was used. The fidelity of this polymerase is 5-15-fold higher than that observed for Taq DNA polymerase. To generate an expression cassette plasmid, PCR fragments of MB3 (full length and truncated fragments) were inserted in *Pichia* expression vectors either directly or using the Original TA Cloning® Kit (Invitrogen), which includes a pCR™II vector for subcloning of PCR fragments. Direct cloning of DNA amplified by either Vent® DNA polymerase or *Pfu* DNA polymerase into the vector pCR™II is difficult, as the cloning efficiency is often very low. This is due to the 3' to 5' exonuclease proofreading activity of Vent® and *Pfu*, which removes the 3' A overhangs that are necessary for TA cloning, leaving blunt ends. The Original TA Cloning® Kit allows these blunt-ended fragments to be cloned. Use of this method eliminates any enzymatic modifications of the PCR product, and does not require the use of PCR primers containing restriction sites. To increase the cloning efficiency further, the Invitrogen protocol was modified as follows. Following amplification with Vent® or *Pfu* (*see* manual for The Original TA Cloning® Kit, protocol for the addition of 3'A-overhangs post amplification, p. 19), rather than placing the vial on ice, as recommended in the kit, the mineral oil in the PCR mixture was immediately removed using Parafilm™. This was accomplished by pouring the PCR mixture onto the Parafilm, and zigzagging the drop down the surface of the Parafilm with a gentle rocking motion until all of the oil had adhered to the Parafilm surface. The reaction mixture, now free of oil, was then collected into a fresh tube. The Invitrogen protocol was then resumed with the

addition of Taq polymerase. This method allowed the difficult cloning of PCR fragments into large expression vectors.

The expression cassette of the integrating vector (Invitrogen) contains the methanol-induced AOX1 promoter and its terminator, flanked by stretches of nucleotides up- and downstream from the AOX1 gene. The *P. pastoris* His4 gene served as an auxotrophic marker. These vectors do not contain a yeast *ori*, hence His$^+$ colonies must correspond to integration of the expression cassette. All PCR fragments of MB3 were inserted in frame with a *Pichia* Kozak consensus sequence (CAAAAAACAA) (Cavenor *et al. Nucleic Acids Res. 19*:3185-3192 (1991); Kozak *Nucleic Acids Res. 15*:8125-8148 (1987); Kozak *Proc. Natl. Acad. Sci. USA 87*:8301-8305 (1990)) to provide the best translation initiation of the MB3 gene. All inserts were placed under the control of the AOX1 promoter to drive expression of the gene of interest. After the ligation of the MB3 fragment in an appropriate expression vector, chemically competent *E. coli* cells were transformed (TOP 10F′) (F′{*pro*AB, *laq*I_q, *lac*ZΔM15, Tn*10* (Tet^R)} *mcr*A, Δ(*mrr-hsd*RMS-*mcr*BC), ϕ80 *lac*ZΔM15, Δ*lac*X74, *deo*R, *rec*A1, *ara*D139, Δ(*ara-leu*)7697, *gal*U, *gal*K, *rps*L(Str^R), *end*A1, *nup*Gλ$^+$). Other strains which may be suitable are DH5α F′, JM109, or any other strain that carries a selectable F′ episome and is *rec*A deficient (*endA* is preferable) (*Pichia* Expression Kit Instruction Manual, Invitrogen). Colonies with an MB3 insert were used for the preparation of CsCl purified maxi-prep of a plasmid DNA for *Pichia* transformation (Sambrook, J. *et al.*, Eds., *Molecular Cloning: A Laboratory Manual*. 2nd. Ed., Cold Spring Harbor Press (1989), pp. 1.42-1.43). Restriction analysis and DNA sequencing (DNA Sequencing Kit, Version 2 (USB)) confirmed that these constructs were correct.

Modification of the starting MB3 sequence was especially useful for intracellular expression of the porin gene (pHIL-D2/MB3 construct). Because the other constructs (pHIL-S1/MB3 and pPIC9/MB3) used for MB3 secretion contained codons optimal for *Pichia* in the leader peptide sequence upstream of the MB3 insert, the initiation of translation was not rate-limiting. In contrast, the

pHIL-D2 vector does not include any leader sequence and the initiation of translation must be started from the rare codons of the MB3 insert. The optimization of this sequence is believed to be responsible for the fact that pHIL-D2/MB3 constructs gave the highest level of MB3 expression of any of the clones tested (Tables 3, 4).

Transformation of yeast cells and DNA analysis of integrants

Plasmid DNA was linearized with single or double (for higher integration efficiencies) digestion, and *P. pastoris* strain GS115 (his4$^-$) was transformed to the His$^+$ phenotype by the spheroplast method using Zymolyase followed by adsorption of transforming DNA and penetration of this DNA through the spheroplast pores into the *Pichia* cells in the presence of PEG and Ca^{+2} (*Pichia* Expression Kit manual, Invitrogen, pp.33-38). By replica plating or patching on Minimal Dextrose (MD: 1.34% yeast nitrogen base (YNB - Difco), $4x10^{-5}$% biotin, 2% dextrose) versus Minimal Methanol (MM: 1.34% YNB, $4x10^{-5}$% biotin, 0.5% methanol), it was possible to determine which His$^+$ transformants also exhibited disruption of the *AOX1* gene. Transformed spheroplasts were seeded on agarose-containing plates using selective growth medium without histidine (MD). At the end of 4-6 days, white separated colonies of yeast transformants had appeared. These colonies were picked up and were seeded on selective methanol-containing medium (MM) for screening of AOX1-disrupted (Mut^s or Mut$^-$) transformants (*Pichia* Expression Kit manual, Invitrogen, p. 60).

Growth of the yeast and methanol induction

Because recombination events can occur in many different ways which affect the level of protein expression (clonal variation), at least 16 verified recombinant clones were screened to determine the level of MB3 expression. These colonies were grown in 5 ml of glycerol-containing Buffered Glycerol-

complex Medium (BMGY: 1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, $4x10^{-5}$% biotin, 1.0% glycerol) (*Pichia* Expression Kit manual, Invitrogen, p. 61) at 30°C in 50 ml 2098 Bluemax tubes (Falcon) in an Innova incubator shaker (New Brunswick Sci.) ("pilot" expression). After 1-2 days when cultures had reached an OD600 = 5-10, the cells were harvested by centrifugation (4000 rpm for 10 minutes at room temperature) and were resuspended in methanol-containing Buffered Methanol-complex Medium (BMMY: 1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, $4x10^{-5}$% biotin, 0.5% methanol) (*Pichia* Expression Kit manual, Invitrogen, p. 61) for the induction of the *AOX1* promoter. To replenish exhausted methanol, 0.5% of fresh methanol was added each day to induced cells. Aliquots of the cells were collected every day for 6 days by centrifugation, and stored (pellets and supernatants separately) at -70°C before examining. The most promising clones were examined for the optimization of protein expression and to scale-up the expression protocol to produce more protein.

Lysis of* P. pastoris *cells, analysis by SDS-PAGE and Western blot analysis

Cells were broken by agitation in breaking buffer (50 mM sodium phosphate, pH 7.4; 1 mM PMSF(phenylmethylsulfonyl fluoride), 1 mM EDTA and 5% glycerol). Equal volumes of acid-washed glass beads (0.5 mm in diameter) were added. The mixture was vortexed for a total of 4 min, 30 sec mixing each, followed by 30 sec on ice. The soluble fraction was recovered by centrifugation for 10 min at 14000 rpm at 4°C. Supernatant (or cell lysate, or fraction of "soluble" proteins) was removed and stored at -70°C, and the residual cell pellet was extracted by vortexing with SDS sample buffer (1% SDS, 5% beta-mercaptoethanol, 10% glycerol, 10 mM EDTA, 0.025% bromophenol blue) followed by boiling for 10 min. Lysates were centrifuged again and the aqueous layer was examined as fraction of "insoluble" or membrane associated proteins.

NOVEX pre-cast 8-16% gradient gels were used for separation of proteins according to the procedure of Laemmli *(Nature 227*:680-685 (1970)). Proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were stained with Coomassie Brilliant Blue R250, or were transferred to polyvinylidene difluoride (PVDF) membrane using a Transblott apparatus (BioRad Laboratories) according to the company specification.

The Western blot procedure was carried out without detergents, using only blocking procedures, as described by Sheng and Schuster (*Bio Technique 13*:704-708 (1992)) with some modifications. This method provides high specificity and sensitivity with a low background. For the transfer, both Western transfer membrane and the SDS-PAGE separating gel were equilibrated with transfer buffer (24mM Tris-HCl/192 mM glycine/ 20% methanol) for 20 minutes prior to electrotransfer. The transfer was performed at 90V and 4°C for 3-4 hours. Transfer of proteins to PVDF membranes was monitored by the transfer of prestained molecular weight markers (BRL).

Immunostaining of proteins was carried out as follows. The transfer membrane was rinsed with TBS (10mM Tris-HCl/.09% NaCl, pH 7.2). The membrane was then incubated in 1% non fat dried milk PBS solution (M-PBS) with .02% sodium azide at 37°C for 3 hours (or at 4°C overnight). The membrane was then washed 3 times with TBS/0.5% BSA (BSA/TBS) and once with TBS. The membrane was then incubated with the primary mouse anti-MB3 antibody (mouse polyclonal antisera against purified OMP class 3) diluted to about 1:4000 in PBS/1%BSA (BSA/PBS), and the membrane was again washed 3 times with TBS/0.5% BSA (BSA/TBS) and once with TBS. The membrane was then incubated in 1% M-PBS at room temperature for 30 minutes with gentle shaking. The membrane was washed 3 times with TBS/0.5% BSA (BSA/TBS) and once with TBS. The membrane was then incubated in the secondary alkaline phosphatase-conjugated anti-mouse antibody (Kirkegaard & Perry Laboratory (KPL), Gaithersburg, MD) diluted 1:4000 in 1% BSA/PBS. The membrane was then washed 2 times with 0.5% BSA/TBS and 3 times with .25% Tween 20 in

PBS. These washing steps differed from those recommended by Sheng and Schuster; the improved protocol provided less background than did the wash steps of the reference, which utilized 6 washes in 0.5% BSA/PBS. The membrane was then incubated in alkaline phosphatase buffer (0.05% M Tris-HCl, pH 9.5; 10 mM $MgCl_2$), followed by incubation in BCIP/NBT substrate solution (KPL). The development was stopped by washing the membrane in PBS/50 mM EDTA. The limit of detection was about 2-5 ng of native MB3 protein.

Results and discussion

The strategy used to insert the cDNA encoding the mature MB3 into expression vectors and the steps using this construct for the transformation of *P. pastoris* are outlined below. First, the MB3 gene is cloned into one of the 4 *Pichia* expression vectors. In the next step, the resulting construct is linearized by digestion with *Not*I or *Bgl*II, and *his4 Pichia* spheroplasts are transformed with the linearized construct. In the following step, a recombination event occurs *in vivo* between the 5´ and 3´*AOX1* sequences in the vector and in the genome, resulting in replacement of the *AOX1* gene with the MB3 gene. Next, the *Pichia* transformants are selected on histidine-deficient medium, on which only cells that have undergone gene replacement can grow. The one-step gene replacement method described for *S. cerevisiae* (Rothstein, *Meth. Enzymol. 101*:202-211 (1983)) was successfully used by Cregg *et al.* (*Biological Research on Industrial Yeast, Vol. II*, Stewart *et al., eds.*, CRC Press, Boca Raton, pp.1-18 (1987)) for the replacement of the *P. pastoris* AOX1 structural gene. Transformation of GS115 with 10 μg of linearized expression vectors (pHIL-D2, pHIL-S1, pPIC9, and pPIC9K) with MB3 insert gave more than 100 colonies in each experiment. Thus, the procedure yielded $>10^2$ His$^+$ colonies per μg DNA, which is comparable to that reported for the best results of *P. pastoris* transformations. These transformants have the ability to grow on histidine-deficient medium (MD-minimal dextrose), and so are His$^+$. About 10-40% of

these recombinants were "methanol slow" (Mut^s -- "methanol utilization slow"), i.e., demonstrated impaired growth on media such as MM (minimal methanol), which contains methanol as the sole carbon and energy source. These His$^+$/Mut^s transformants are a result of the replacement of the AOX1 structural gene with the MB3 expression cassette containing the His$^+$ gene via a double crossover event. Recombination events may also occur as integration or insertion (single crossover events) of the expression cassette into the 5' or 3' AOX1 region, which leaves the AOX1 gene intact. Among the His$^+$/Mut^s clones, 25-35% were positive, MB3-expressing transformants (Table 2). The reason that the *AOX1*-deleted transformants grow at all on methanol medium is due to low-level expression of alcohol oxidase activity by the *AOX2* gene product. Analysis of DNA isolated from these "positive" recombinants using PCR with 5' *AOX1*, 3' *AOX1*, 5' MB3, 3' MB3 and other specific primers, indicated that the *AOX1* structural gene was indeed replaced by the fragment containing the MB3 and *HIS4* genes. Analysis of the DNA isolated from His$^+$/Mut$^+$ transformants indicated that the *AOX1* structural gene was intact and that the entire vector containing His4 DNA had integrated elsewhere. Among 39 *AOX1*-disrupted transformants that expressed MB3, no His$^+$/Mut$^+$ transformants were found, indicating preference for the *AOX1* replacement mode of integration.

The results of immunoblot analysis of 84 *Pichia* transformants indicated that one may express the MB3 protein using all of the constructed recombinant plasmids, pHIL-D2/MB3, pHIL-S1/MB3, pPIC9/MB3, and pPIC9K/MB3 (Table 3). Thirty-nine clones were isolated that expressed the MB3 protein. Antigenic specificity of expressed MB3 protein was examined and was confirmed by Western blot analysis using monoclonal and polyclonal antibodies raised against wild type *N. meningitidis* OMP class 3. These results led to the conclusion that all of the expression vectors were correctly constructed, and that the transformations of *Pichia* spheroplasts were properly performed.

The amount of expressed MB3 was determined by densitometric scanning of the Coomassie brilliant blue stained protein bands fractionated by SDS-PAGE

using a Model GDS-7500 scanning densitometer (UVP Life Sci.) or Model IS-1000 densitometer (Alpha Innotech Corp.). Purified OMP class 3 extracted wild type of *N. meningitidis* was used as a standard. Based on the results (summarized in Table 3), the level of protein expression was estimated to be moderate to high.

The optimization of the translation elongation rate for the expression of the MB3 gene (see Materials and Methods, above) was very useful. The modification of the starting MB3 sequence was especially effective for intracellular expression of the porin gene (pHIL-D2/MB3 construct). Because other constructs (pHIL-S1/MB3 and pPIC9/MB3, both used for MB3 secretion) contained codons optimal for *Pichia* in the leader peptide sequence upstream of the MB3 insert, the initiation of translation of these cassettes was not rate-limiting. In contrast, the pHIL-D2/MB3 construct did not include a leader sequence, and so without codon optimization, translation would have had to have been initiated at rare codons of the MB3 insert. The codon-optimized pHIL-D2/MB3 construct, when transformed into *Pichia* chromosomal DNA, provided the highest level of MB3 expression of all the other mentioned MB3 expression constructs (Tables 3 and 4). Thus, this modification of the translation start sequence of MB3 appears to be responsible for the high yield of expressed protein in pHIL-D2/MB3 constructs.

The level of MB3 expression by the best clones (*Pichia* transformed with the pHIL-D2/MB3 construct) was in the range of 0.1-0.6 g per 1L of cell suspension, or 1-3 mg per g of cell pellet (Table 3, Fig. 12). Such efficiency of expression in yeast has been reported for many of the following manufactured proteins: hepatitis B surface antigen (0.3 g/L), superoxide dismutase (0.75 g/L), bovine and human lysozyme (0.3 and 0.7 g/L, respectively), human and mouse epidermal growth factors (0.5 and 0.45 g/L respectively), human insulin-like growth factor (0.5 g/L), human interleukin-2 (1.0 g/L), aprotinin analog (0.8 g/L), Kunitz protease inhibitor (1.0 g/L), etc. (Cregg *et al.*, *Biotechnology*, *11*:903-906, Table 1 (1993)).

It should be emphasized that all of the previously listed levels of expression for manufactured proteins are the result of production of these proteins during fermentation in high cell density fermentors. MB3 was expressed utilizing only shake flask cultures which, as a rule, provide much lower expression levels than does fermentation. Recently reported observations lead one to expect a much higher yield (a 5-10 fold or greater increase) of MB3 in a fermenter (Cregg *et al.*, 1993). *P. pastoris* adapts well to being scaled up from shake flask to high density fermentor cultures. In addition, where *AOX*-deleted *Pichia* strains are used for fermentation, production of foreign proteins can be optimized by first causing rapid growth, and then adding methanol to induce protein production while minimizing additional cell growth. The long amount of time needed to produce proteins when *Pichia* is growing on methanol can be reduced by applying one of several mixed-feed fermentation strategies (Siegel *et al.*, *Biotechnol. Bioeng. 34*:403-404 (1989); Brierley *et al.*, Int. Patent Application No. WO 90/03431 (1989); Brierly *et al.*, *Biochem. Eng. 589*:350-362 (1990); Siegel *et al.*, Int. Patent Application No. WO 90/10697 (1990)).

Another promising aspect of the expression levels of MB3 protein in *Pichia* is that the results were similar for all examined clones. As other investigators have found that in shake flask induction the level of expression is proportional to the number of copies of inserted gene of interest (Clare *et al.*, 1991), it can be deduced that all of the MB3 clones tested were single-copy chromosomal integrants, and thus that no *Pichia* recombinants with multiple integrated copies of the MB3 fragment were isolated.

An important factor in obtaining high levels of expression using *P. pastoris* is the ability to obtain recombinants with multicopy transplacement or integration (Romanos *et al.*, *Vaccine 9*:901-906 (1991); Clare *et al.*, *Bio/Technology 9*:455-460 (1991); Clare *et al.*, *Gene 105*:205-121 (1991)). . Multicopy transformants have been found to be surprisingly stable over multiple generations during growth and induction in high cell density fermentations. Since this multiple gene insertion event occurs at a low frequency during

spheroplast transformation, a special dot blot screening of a number of recombinants is used (Scorrer *et al.*, *Bio/Technology 12*:181-184 (1993)). An alternative to screening for spontaneous multiple insertion events is to insert multiple copies of the gene(s) of interest into *Pichia* expression vector pAO815, which has recently been constructed by Invitrogen for this purpose.

Before attempting to express MB3, the protein was evaluated to determine if any of the factors believed to reduce expression levels were present. One of the factors which can reduce expected high-level accumulation of a protein is proteolytic stability. It is now known that highly expressed proteins are devoid of good PEST sequences. PEST sequences contain proline (P), glutamic acid (E), serine (S) and threonine (T), and are found in all rapidly degraded eukaryotic proteins of known sequence; such proteins have been implicated as favored substrates for calcium-activated proteases (Rogers *et al.*, *Science 234*:364-369 (1986)). Proteins that are expressed at high levels in yeast do not contain a so-called "good" PEST sequence (having a score >5 as calculated by the algorithm developed by Rogers *et al.* (1986)), which leads to susceptibility to proteolysis, nor do they contain the pentapeptide sequences XFXRQ or QRXFX (X=any amino acid), which are selective for degradation of cytoplasmic proteins by the lysosomal pathway (Dice, J.F., *Fed. Am.Soc. Exp. Biol. (FASEB) J. 1*:349-357 (1987)). Proteins that are expressed at high levels in yeast do not contain these pentapeptide sequences. Computer analysis of the MB3 sequence identified a "poor" but not "good" PEST region (13-32aa) having the sequence (ETSRSVFHQNGQVTEVTTAT. According Rogers *et al.* (1986) such a poor PEST sequence weakly influences the proteolytic stability of eukaryotic proteins. Thus, one of the factors which leads to proteolysis is not present in MB3.

MB3 also does not contain the highly conserved pentapeptide sequences mentioned above. The sequence RQSEI (75-79aa) is present in MB3: this sequence displays some homology to the degradation pentapeptide QRXFX, but is not believed to greatly destabilize MB3.

The nature of the NH_2-terminal amino acid residue can also be an important factor in the susceptibility of a protein to degradation. Varshavsky *et al.* have demonstrated that the presence of certain amino acids at the NH_2-terminus provide a stabilizing effect against rapid degradation by ubiquitin-mediated pathways (the N-end rule pathway) (Varshavsky *et al. Yeast Genetic Engineering*, Butterworths, pp. 109-143 (1989)). Most proteins that are expressed at high levels in yeast have a stabilizing amino-terminus amino acid residue (A, C, G, M, S, T or V). Examples of such proteins include human superoxide dismutase, human tumor necrosis factor, phosphoglycerate kinase from *S. cerevisiae*, invertase from *S. cerevisiae*, alcohol oxidase from *P. pastoris*, and extracellular alkaline protease from *Y. lipolytica* (Sreekrishna *et al.*, *Biochemistry 28*:4117-4125 (1989)). Although MB3 is expressed well in yeast, the NH_2-terminal aspartic acid (D) of MB3 does not provide a stabilizing effect against rapid degradation by ubiquitin-mediated pathways.

It is possible that the NH_2-terminal aspartic acid of MB3 will play a role in the level of MB3 produced from *Pichia* in large scale production. Replacing the first amino acid of MB3 with one of the amino acids known to stabilize the NH_2-terminus of proteins, mentioned above, could improve the level of MB3 production.

It was decided to proceed with experiments attempting to express MB3 in yeast, as most of the factors known to reduce expression levels were not present in MB3.

The best expression of MB3 was provided by *Pichia* clones transformed with the pHIL-D2/MB3 expression cassette (Tables 3 and 4). This pHIL-D2 vector generated intracellular expression of complete, monomeric, non-fusion, non-secreted MB3 with an expected MW of about 34 kDa. These clones provided the highest level of expression of MB3, up to 600 mg/L or 3 mg per g of wet cell pellet (Table 4). About 90-95% of this product was insoluble, membrane-associated material, *i.e.*, material which sediments upon centrifugation for 5 min at 10,000g, and that can be extracted by treatment with SDS-

containing buffer (PAGE sample buffer) followed by boiling. The protein can then be renatured to a conformation that can be easily recognized by an anti-meningococcal OMP class 3 antibody.

Induction of pHIL-D2/MB3 constructed clones with methanol resulted in the rapid expression and fast accumulation of intracellular MB3. After 24 hours of a methanol induction, the level of expressed MB3 was estimated at not less than 80% of maximal, which was reached after 5-6 days.

The pHIL-D2/MB3-containing *Pichia* recombinant is the most promising for commercial production. This clone provides relatively high levels of expression which could be significantly improved by using multiple-copy recombinants, and by producing the protein in a fermentor. The fact that MB3 is rapidly produced also provides an advantage for large scale manufacture.

MB3 expressed in an intracellular form was purified by a denaturation/renaturation protocol, followed by gel filtration and ion exchange chromatography. The resultant purified protein exhibits an elution profile on size exclusion chromatography that resembles the recombinant class 3 protein overexpressed in *E. coli*. MB3 expressed by either *E. coli* or *P. pastoris* co-elutes with the native wild-type counterpart, indicating that MB3 expressed by either *E. coli* or *P. pastoris* refolds and oligomerizes, achieving full native conformation (Figs. 14A and 14B).

Both the native (*Pichia*) secretion signal (PHO1) and the alpha-factor signal sequence from *S. cerevisiae* were tested for targeting expressed porin to the secretary pathway. Unexpectedly, the shorter PHO1 leader was more effective for causing MB3 secretion. The pHIL-S1 *Pichia* transfer vector includes a sequence encoding the 2.5 kDa PHO1 leader peptide, a secretion signal peptide of *P. pastoris*. In the pHIL-S1/MB3 construct, the sequence encoding MB3 was inserted downstream of the PHO1 leader sequence. 40-50% of the 36.5 kDa expressed fusion protein PHO1/MB3 produced by pHIL-S1/MB3 clones was properly cleaved to generate a 34 kDa MB3 monomer (Tables 2 and 3), and 5-10% of expressed soluble porin was secreted. The pPIC9 and pPIC9K

Pichia transfer vectors include a sequence encoding the 10 kDa alpha-factor leader derived from *S. cerevisiae*. *Pichia* clones transformed by pPIC9/MB3 or pPIC9K/MB3 did not secrete porin. These recombinants expressed a 44 kDa alpha-factor prepro/MB3 fusion protein well, but no evidence of correct cleavage and processing was observed. Improved secretion of expressed MB3 was not obtained by using its 3' truncated fragment fused with either PHO1 leader or alpha-factor leader peptides.

Example 10. Isolation, purification and characterization of MB3 protein expressed as a secretory protein

Yeast cells cultures harboring the expression vector containing the gene for MB3 (pHIL-S1-pNV318) were configured to isolate the protein as soluble secreted material). The supernatant was clarified by precipitation with 20% ethanol (v/v) to remove contaminating yeast culture impurities. The supernatant was then precipitated with 80% ethanol (v/v). The resulting pellet was washed with TEN buffer (Tris HCl, pH 8.0, 100 mM NaCl and 1 mM EDTA), in order to remove other hydrosoluble contaminating secreted proteins. The pellet containing MB3 was dissolved in an aqueous solution of detergent (solubilizing buffer), comprised of TEN buffer with 5% Z 3-14. The solution was applied to a Hi-Trap Q Sepharose ion exchange column (1 ml) (Pharmacia) equilibrated in 50 mM Tris, 0.2 M NaCl and 1.0 mM EDTA (pH 8.0). A gradient of 0.2-1.0 M NaCl was applied, and MB3 protein eluted as a single peak.

Example 11. Isolation, purification and characterization of MB3 protein expressed as an insoluble-membrane bound protein

Yeast cells cultures harboring the expression vector containing the gene for MB3 (pHILD-2--pNV322) (see Table 3) were resuspended in breaking buffer

(i.e., 50 mM sodium phosphate buffer, pH 7.4, 1 mM EDTA, and 5% glycerol), to a concentration equivalent to 50-100 ODs. The suspension was added to the same volume of acid treated glass beads. The suspension was lysed using a Minibead-Beater (Biospec Products, Bartlesville, OK), in 8 consecutive cycles of 1 min each, followed by 1 min on ice, between each cycle. As an alternative procedure, the lysis process was facilitated by the addition of Zymolase to the breaking buffer. The suspension was transferred to a glass sintered filter to separate the glass beads, and the cell suspension was collected in the filtrate. The beads were further washed and the filtrates combined. The suspension was then centrifuged at 12,000 rpm for 15 min at 4°C. A series of consecutive washing steps was applied to the resultant pellet, consisting of the following: (a) TEN (Tris HCl, pH 8.0, 100 mM NaCl, and 1 mM EDTA) containing 0.5% deoxycholate; (b) TEN containing 0.1% SDS and 1% Nonidet, after which the suspension was rotated for 30 min at 25°C; (c) washing with TEN buffer; and (d) washing with TEN buffer containing 5% Z 3-14, under rotation overnight at 4°C. Each washing step was followed by centrifugation at 12,000 rpm for 10 min at 4°C to collect the pellet for the following step. As an alternative method of washing the pellet, the suspension was passed through an 18 gauge needle in lieu of rotation in steps (b) and (d). Finally, the MB3 was extracted with 8M urea, or 6M guanadinium HCl, and the extract was sonicated for 10 min, using a water bath sonicator. The extract was clarified by centrifugation (12,000 rpm, for 10 min at 4°C), the same volume of a 10% aqueous solution of 3,14-zwittergen (Calbiochem) was added and the solution thoroughly mixed. The solution was again sonicated for 10 min. Any residual material was removed by centrifugation. This mixture was then applied to a Sephacryl S-300 (5x100 cm) column (Pharmacia) equilibrated in a buffer comprised of 0.1 M Tris-HCl, 0.2 M NaCl, 10 mM EDTA, 20 mM $CaCl_2$ and 0.05% Z 3-14 (pH 8.0). Fractions containing class 2 protein were identified by SDS-PAGE, pooled, and applied to a Hi-Trap Q Sepharose ion exchange column (1 ml) (Pharmacia) equilibrated in 50 mM Tris, 0.2 M NaCl and 1.0 mM EDTA (pH 8.0). A gradient of 0.2-1.0 M

NaCl was applied and MB3 protein eluted as a single peak. Figures 14A, 14B and 15 depict the elution profile of purified MB3 in a Sepharose 12 (Pharmacia) connected to an HPLC (Hewlett Packard, model 1090). Based on the comparison with the native wild-type class 3 protein, as well as calibration using molecular weight standards, the elution profile is indicative of trimeric assembly.

Example 12. *Preparation of GAMP-TT Conjugate*

12.1 Preparation of NMA polysaccharide for conjugation. *N. meningitidis* group A (NMA) strain 604 A was grown in modified Franz medium (Franz, I. D., *J. Bact. 73:*757-761 (1942). Precipitation of the polysaccharide as a cationic detergent complex followed by fractional precipitation with ethanol provided the high molecular NMA capsular polysaccharide. The high molecular weight polysaccharide was further purified by ultra filtration. Partial hydrolysis of the polysaccharide with 100 mM sodium acetate buffer pH 5.0 at 70°C yielded a low molecular weight polysaccharide in the range of 10,000-20,000 daltons. The free reducing terminal residue of the polysaccharide was reduced with $NaBH_4$ in the cold to preserve O-acetyl substituents and then oxidized with sodium periodate to generate terminal aldehyde groups. The oxidized polysaccharide was the purified and fractionated by size exclusion chromatography to provide activated group A meningococcal polysaccharide (GAMP) of average molecular weight about 13,000 daltons.

12.2 Preparation of GAMP-TT conjugate. Tetanus toxoid (Serum Statens Institute, Denmark) was first purified to its monomeric form (mw 150,000) by size exclusion chromatography using a Superdex G-200 column (Pharmacia). Freeze-dried tetanus toxoid monomer (1 part by weight) and oxidized GAMP (2.5 part by weight) were dissolved in 0.2 M phosphate buffer pH 7.5. Recrystallized $NaBH_3CN$ (1 part) was added and the reaction mixture incubated at 37°C for 4 days. The conjugate was purified from the free components by size exclusion chromatography using a Superdex G-200 column

(Pharmacia), and PBS containing 0.01% thimerosal as an eluent. Purified GAMP-tetanus toxoid conjugate was stored at 4°C in this buffer. The polysaccharide content of the conjugate based on phosphorus analysis (Chen assay) was about 18-20% by weight.

Example 13. *Preparation of GCMP-TT Conjugate*

13.1 ***Preparation of NMC polysaccharide for conjugation.*** The capsular polysaccharide was isolated from the growth medium of *Neisseria meningitidis* group C (NMC) strain C 11. This strain was grown in modified Franz medium. The NMC polysaccharide (group C meningococcal polysaccharide (GCMP)) was isolated from the culture medium by cetavlon precipitation as described for the GAMP. Native GCMP was O-deacetylated with base and depolymerized by oxidative cleavage with $NaIO_4$ to an average molecular weight of 10,000-20,000. The cleaved polysaccharide was sized and purified by gel filtration chromatography to provide a highly purified product of average molecular weight about 12,000 daltons and having aldehyde groups at both termini.

13.2 ***Preparation of GCMP-TT conjugate.*** Tetanus toxoid monomer (1 part) and solid oxidized GCMP (1 part) were dissolved in 0.2 M phosphate buffer pH 7.5 and incubated at 37°C with 1 part of recrystallized $NaBH_3CN$ for 4 days. The conjugate was purified from its free components by gel filtration chromatography on Superdex G-200 using PBS containing 0.01% thimerosal as eluent. The purified conjugate was stored at 4°C prior to being formulated for animal studies. The content of the polysaccharide in the conjugate was 33% based on its sialic acid content as measured by the Svennerholm resorcinol assay (*Biochim. Biophys. Acta 244:*604-611 (1957).

Example 14. *Preparation of N-Propionyl Group B Meningococcal Polysaccharide-rPorB Conjugate*

14.1 Preparation of Neisseria rPorB. Expression of class 3 *N. meningitidis* porin protein (PorB) in *E. coli* and purification of porin gene products is described *supra*. The recombinant rPorB protein was purified by using a sephacryl S-300 molecular sieve column equilibrated with 100 mM Tris-HCl, 200 nM NaCl, 10 mM EDTA, 0.05% Zwittergen 3, 14 (Calbiochem. La Jolla, CA), 0.02% sodium azide pH 8.0. The protein fractions as measured by their OD_{280} eluting with an apparent molecular weight of trimers were pooled and diafiltered against 0.25 M HEPES, 0.25 M NaCl, 0.05% Zwittergen 3, 14 pH 8.5, to a concentration of 10-12 mg/ml.

14.2 Preparation of N-propionylated Group B Meningococcal Polysaccharide (GBMP). The N-propionylated GBMP and its oxidized form were prepared as described in U.S. Patent No. 4,727,136 and EPO 0504202 , both of which are fully incorporated by reference herein.

14.3 Preparation of N-Pr-GBMP-rPorB conjugate. To 10 mg of oxidized N-Pr-GBMP of average molecular weight 12,000 was added 33 µl of a 12 mg/ml of rPorB protein in 0.25 M HEPES, 0.25% M NaCl, 0.05% Zwittergen 3, 14, pH 8.5. The solution was mixed until all solid dissolved and 6.5 mg of recrystallized $NaBH_3CN$ was added. The solution was incubated at 37°C for 4 days and the conjugate was purified from the mixture by using a Superdex G-200 column (Pharmacia) equilibrated with PBS -0.0% thimerosal. Protein fractions were combined and stored at 4°C. The conjugates were analyzed for their sialic acid content by the resorcinol assay and for protein with the Pierce Coomassie Plus assay. The resulting conjugate had a polysaccharide content of about 20-25% and is devoid of any pyrogens as measured by the LAL and rabbit pyrogenicity tests.

Example 15. Analysis of Conjugates by Capillary Electrophoresis

15.1 System and method. Analysis was performed by Capillary Zone Electrophoresis on a Beckman 2000 Series CE system (Beckman Instruments Inc., Fullerton, CA) using an untreated fused silica capillary of dimensions 47 cm total length (40 cm effective length) by 50 µm i.d. (375 µm o.d.) and 0.4N borate buffer, pH 10.2 as electrolyte (Hewlett Packard, Palo Alto, CA). System control and data acquisition was performed using Beckman Gold system software. The voltage was set at 25 KV and the detector was set to 200 nm detection wavelength. The capillary temperature was set to 20°C. The capillary was conditioned between runs with a high pressure rinse for 2.0 minutes with 0.1M sodium hydroxide followed by 2.0 minutes with deionized water. All samples were pressure injected. All buffer and sample media were filtered through an appropriate 0.2 µm membrane filter and degassed prior to use.

15.2 Analysis of Conjugates. After purification the conjugates were concentrated by ultrafiltration through an Amicon Centricon-3 concentrator (Amicon, Inc., Beverly, MA). Meningococcal polysaccharide and tetanus toxoid monomer calibration samples were prepared in deionized water at a concentration of 0.25 mg/ml and 0.28 mg/ml, respectively. The method was determined to be selective for the glycoprotein and conjugate components with adjacent components being completely separated (Rs>1.5), as demonstrated in the electropherograms of the polysaccharides and protein spiked glycoprotein conjugates (Fig. 20 and Fig. 21). Fig. 20 shows the GAMP-TT conjugate spiked with GAMP and TT-monomer conjugate components, while Fig. 21 shows the GCMP-TT conjugate spiked with GCMP and TT-monomer conjugate components. The lower limit of detection (LLD) for the free form polysaccharide and protein components for the method was determined to be in the subnanogram level. A lower limit of quantitation (LLQ) of approximately 0.6 ng was obtained for the free form of each component. A linear response was obtained for the selected total mass of each component. A linear response was obtained for the

selected total mass range of 0.6-2.6 ng and 0.6-2.4 ng for the polysaccharide and protein, respectively, with a coefficient of determination of 0.99 for both curves. Using this CZE based assay, analysis of a meningococcal polysaccharide-tetanus toxoid conjugate indicated a free polysaccharide content of less than about 5% and a free protein content of less than about 2%.

Example 16. Immunization and Immunoassays

16.1 Trivalent conjugate vaccine formulation. Each individual conjugate component (A, B, C) was absorbed onto Aluminum hydroxide ($Al(OH)_3$) Alhydrogel (Superfos, Denmark) at a final Al concentration of 1 mg/ml of the trivalent vaccine. Three vaccines were formulated in which the doses of each conjugated polysaccharide varied. Formulations had either about 2 μg of each A, B, and C conjugated polysaccharide; or about 2 μg A conjugated polysaccharide, about 5 μg B conjugated polysaccharide and about 2μg C conjugated polysaccharide; or about 5 μg of each A, B, and C conjugated polysaccharide per dose of 0.2 ml of PBS, 0.01% thimerosal.

16.2 Immunization. Female Balb/c mice (Charles River Laboratories) 4-6 weeks old, were injected i.p. at days 0, 28, and 42. Bleeds were performed at days 0, 14, 28, and 42, and mice were finally exsanguinated at day 52. Sera were stored at -70°C prior to serological analysis.

16.3 Immunoassays:

ELISAs: Antibody titers to each A, N-propionylated B and C polysaccharides were determined by ELISA using the corresponding HSA conjugates as coating antigen (Figs. 22, 23, and 24). Antibody titer was defined as the x-axis intercept of the linear regression curve of absorbance vs. absorbance x dilution factor.

Bactericidal Assays: Bactericidal assays were performed using baby rabbit serum as a source of complement and *N. meningitidis* strains H 44/76 (Serotype 15), C11 and A1 respectively used as group B meningococcal, group C meningococcal, and group A meningococcal organisms in this assay (Figs. 25,

26, and 27). Bactericidal titer was defined as the serum dilution producing 50% reduction in viable counts.

Having now fully described this invention, it will be understood by those of ordinary skill in the art that the invention can be practiced within a wide and equivalent range of conditions, formulations and other parameters without affecting the scope of the invention or any embodiment thereof. All patents and publications cited herein are fully incorporated by reference herein in their entirety.

Table 1. ELISA and Bactericidal Titers of Group B Meningococcal Conjugate Vaccines (N-Pr GBMP-Protein)

Vaccine	Adjuvant	ELISA Titer	Bactericidal Titer
N-Pr GBMP-TT	Saline	5,400	0
	$Al(OH)_3$	13,000	0
	ST[1]	17,000	0
	CFA[2]	40,000	800
N-Pr GBMP-PP	Saline	20,000	500
	Saline	22,000	150
	Saline	39,000	960
	$Al(OH)_3$	93,000	200
	$Al(OH)_3$	166,000	>3,200
	$Al(OH)_3$	130,000	1,200
	ST	53,000	1,000
	ST	29,000	1,700
	ST	72,000	1,500
N-Pr GBMP	Saline	>100	0
	$Al(OH)_3$	>100	0
	ST	>100	0
PP	Saline	>100	0
	$Al(OH)_3$	>100	0
	ST	660	0

[1]ST = Stearyl tyrosine.

[2]CFA = Complete Freund's Adjuvant

Table 2. Efficacy of a transformation of yeast (Pichia) cells

Construct	Number of analyzed transformants	MB3 expressed transformants	
		Number of positive	% from total
pHIL-D2 / MB3	32	9	28
pHIL-S1 / MB3	23	8	35
pPIC9 / MB3	16	4	25
pPIC9K / MB3	16	5	31

Table 3. Expression of MB3 porin protein with recombinant *Pichia pastoris*

Code AMVAX	Clone	Vector	Level of expression mg / g	mg / L	Secretion
pnv 311	S1/MB3/3/s	pHIL-S1	ND	20 - 30	0
pnv 312	S1/MB3/5/s	pHIL-S1	ND	30 - 40	0
pnv 313	S1/MB3/7/s	pHIL-S1	ND	30 - 40	0
pnv 314	S1/MB3/12/s	pHIL-S1	ND	20 - 30	5 - 10
pnv 315	S1/MB3/15/s	pHIL-S1	ND	20 - 30	0
pnv 316	S1/MB3/18/s	pHIL-S1	ND	80 - 100	5 - 10
pnv 317	S1/MB3/22/s	pHIL-S1	ND	50 - 60	5 - 10
pnv 318	S1/MB3/23/s	pHIL-S1	ND	300 - 400	5 - 10
pnv 321	D2/MB3/1-7/s	pHIL-D2	2.4	480	0
pnv 322	D2/MB3/2-1/s	pHIL-D2	3.0	600	0
pnv 323	D2/MB3/2-6/s	pHIL-D2	1.7	340	0
pnv 324	D2/MB3/2-8/s	pHIL-D2	1.6	320	0
pnv 325	D2/MB3/4-1/s	pHIL-D2	1.7	340	0
pnv 326	D2/MB3/4-3/s	pHIL-D2	2.4	480	0
pnv 327	D2/MB3/4-4/s	pHIL-D2	2.4	480	0
pnv 328	D2/MB3/4-5/s	pHIL-D2	2.4	480	0
pnv 329	D2/MB3/4-26/s	pHIL-D2	2.4	480	0
pnv 341	P9/MB3/1-46/s	pPIC-9	ND	10 - 20	0
pnv 342	P9/MB3/1-261/s	pPIC-9	ND	80 - 100	0
pnv 343	P9/MB3/1-263/s	pPIC-9	ND	20 - 30	0
pnv 344	P9/MB3/1-268/s	pPIC-9	ND	20 - 30	0
pnv 345	9K/MB3/Tr/3-4/s	pPIC-9K	ND	150 - 200	5
pnv 346	9K/MB3/Tr/3-5/s	pPIC-9K	ND	100 - 150	0
pnv 347	9K/MB3/Tr/3-6/s	pPIC-9K	ND	100 - 150	0
pnv 348	9K/MB3/Tr/3-8/s	pPIC-9K	ND	80 - 100	0
pnv 349	9K/MB3/Tr/3-9/s	pPIC-9K	ND	80 - 100	0

Code AMVAX	Clone	Vector	Level of expression mg / g	mg / L	Secretion
pnv 350	9K/MB3/6-1/s	pPIC-9K	ND	150 - 200	0
pnv 351	9K/MB3/6-2/s	pPIC-9K	ND	100 - 150	0
pnv 352	9K/MB3/6-3/s	pPIC-9K	ND	100 - 150	0
pnv 353	9K/MB3/6-5/s	pPIC-9K	ND	80 - 100	0
pnv 354	9K/MB3/6-9/s	pPIC-9K	ND	80 - 100	0
pnv 355	9K/MB3/8-22/s	pPIC-9K	ND	150 - 200	0
pnv 356	9K/MB3/9-5/s	pPIC-9K	ND	80 - 100	0
pnv 357	9K/MB3/10-20/s	pPIC-9K	ND	80 - 100	0
pnv 358	9K/MB3/10-33/s	pPIC-9K	ND	80 - 100	0
pnv 359	9K/MB3/Tr/11-	pPIC-9K	ND	150 - 200	0
pnv 360	9K/MB3/Tr/11-	pPIC-9K	ND	150 - 200	0
pnv 361	9K/MB3/Tr/11-	pPIC-9K	ND	80 - 100	0
pnv 362	9K/MB3/Tr/11-	pPIC-9K	ND	80 - 100	0

Table 4. The expression of MB3 by recombinant clones with different expression cassettes. The main characteristic of the best clones.

CODE:	pnv318 s1/MB3/ 23/s	pnv322 D1/MB3/2-1/s	pnv345 9K/MB3/Tr/3-4/s	pnv350 9K/MB3/6-1/s
CHARACTERISTIC:				
Expression vector	pHIL-S1	pHIL-D2	pPIC 9K	pPIC 9K
Fused leader peptide	PHO1 (2.5kDa)	NO	a-factor(10kDa)	a-factor(10KDa)
Promoter for MB3	AOX1	AOX1	AOX1	AOX1
Size of expr. protein(s)	34.0; 37.5kDa	34.0kDa	43kDa	44kDa
Cleavage (Processing)	Cleavage (40-50%)	NO	NO	NO
Secretion	Weak, <10%	NO	NO	NO
MB3 degradation	<10%	<10%	<10%	<10%
Express level(mg/g)	2.0	3.0	2.0	1.5
Expression Level (mg/L)	300.0	600.0	150.0	150.0
Cytosol localization	60-70%	5-10%	50%	50%
Membrane association	30-40%	90-95%	50%	50%
Solubility	Partly soluble	Insoluble	Partly soluble	Partly soluble

Table 5. Codon Usage for *Pichia pastoris* and MB3

Pichia pastoris codon usage														
TTT	phe	F	11	TCT	ser	S	13	TAT	tyr	Y	6	TGT	cys	C	5
TTC	phe	F	5	TCC	ser	S	9	TAC	tyr	Y	8	TGC	cys	C	2
TTA	leu	L	3	TCA	ser	S	2	TAA	OCH	Z	-	TGA	OPA	Z	-
TTG	leu	L	26	TCG	ser	S	3	TAG	AMB	Z	-	TGG	trp	W	3
CCT	leu	L	4	CCT	pro	P	6	CAT	his	H	-	CTG	arg	R	4
CTC	leu	L	1	CCC	pro	P	5	CAC	his	H	3	CGC	arg	R	2
CTA	leu	L	4	CCA	pro	P	4	CAA	gln	Q	12	CGA	arg	R	-
CTG	leu	L	8	CCG	pro	P	1	CAG	gln	Q	1	CGG	arg	R	2
ATT	ile	I	8	ACT	thr	T	17	AAT	asn	N	9	AGT	ser	S	6
ATC	ile	I	7	ACC	thr	T	5	AAC	asn	N	4	AGC	ser	S	1
ATA	ile	I	3	ACA	thr	T	5	AAA	lys	K	15	AGA	arg	R	6
ATG	ile	M	4	ACG	thr	T	1	AAG	lys	K	14	AGG	arg	R	6
GTT	val	V	15	GCT	ala	A	17	GAT	asp	D	15	GGT	gly	G	13
GTC	val	V	6	GCC	ala	A	6	GAC	asp	D	12	GGC	gly	G	5
GTA	val	V	2	GCA	ala	A	9	GAA	glu	E	23	GGA	gly	G	6
GTG	val	V	10	GCG	ala	A	1	GAG	glu	E	11	GGG	gly	G	-

Outer membrane group B porin protein class 3 (MB3) codon usage														
TTT	phe	F	2	TCT	ser	S	8	TAT	tyr	Y	4	TGT	cys	C	-
TTC	phe	F	11	TCC	ser	S	7	TAC	tyr	Y	11	TGC	cys	C	-
TTA	leu	L	1	TCA	ser	S	-	TAA	OCH	Z	1	TGA	OPA	Z	-
TTG	leu	L	11	TCG	ser	S	4	TAG	AMB	Z	-	TGG	trp	W	4
CCT	leu	L	2	CCT	pro	P	2	CAT	his	H	2	CTG	arg	R	4
CTC	leu	L	3	CCC	pro	P	3	CAC	his	H	7	CGC	arg	R	8
CTA	leu	L	-	CCA	pro	P	-	CAA	gln	Q	10	CGA	arg	R	-
CTG	leu	L	7	CCG	pro	P	-	CAG	gln	Q	4	CGG	arg	R	1
ATT	ile	I	5	ACT	thr	T	5	AAT	asn	N	6	AGT	ser	S	-
ATC	ile	I	7	ACC	thr	T	7	AAC	asn	N	12	AGC	ser	S	9
ATA	ile	I	-	ACA	thr	T	-	AAA	lys	K	21	AGA	arg	R	1
ATG	met	M	2	ACG	thr	T	1	AAG	lys	K	2	AGG	arg	R	-
GTT	val	V	10	GCT	ala	A	4	GAT	asp	D	9	GGT	gly	G	14
GTC	val	V	5	GCC	ala	A	7	GAC	asp	D	12	GGC	gly	G	23
GTA	val	V	9	GCA	ala	A	9	GAA	glu	E	11	GGA	gly	G	1
GTG	val	V	7	GCG	ala	A	2	GAG	glu	E	4	GGG	gly	G	-

What Is Claimed Is:

1. A method for the high level expression of the outer membrane meningococcal group B porin protein or a fusion protein thereof in yeast, comprising:

(a) ligating into a plasmid having a selectable marker a gene coding for a protein selected from the group consisting of:

(i) a mature porin protein

(ii) a fusion protein which is a mature porin protein fused to a yeast secretion signal peptide;

wherein said gene is operably linked to a yeast promoter;

(b) transforming said plasmid containing said gene into a yeast strain;

(c) selecting the transformed yeast by growing said yeast in a culture medium allowing selection of said transformed yeast;

(d) growing the transformed yeast, and

(e) inducing expression of said protein to give yeast containing said protein;

wherein the protein so expressed comprises more than about 2% of the total protein expressed in said yeast.

2. The method according to claim 1, wherein the protein so expressed comprises about 3-5% of the total protein expressed in said yeast.

3. The method according to claim 1, wherein said mature porin protein is the *Neisseria meningitidis* mature outer membrane class 3 protein from serogroup B.

4. The method according to claim 1, wherein said yeast promoter is the AOX1 promoter.

5. The method according to claim 1, wherein said yeast secretion signal peptide is selected from the group consisting of the secretion signal of the *S. cerevisiae* α-mating factor prepro-peptide and the secretion signal of the *P. pastoris* acid phosphatase gene.

6. The method according to claim 1, wherein said plasmid is selected from the group consisting of pHIL-D2, pHIL-S1, pPIC9 and pPIC9K.

7. The method according to claim 1, wherein said gene comprises a nucleotide sequence that incorporates codons optimized for yeast codon usage.

8. The method according to claim 7, wherein said codons optimized for yeast codon usage are in the 5′ region of said gene.

9. The method according to claim 8, wherein said 5′ region of said gene is the nucleotide sequence:

5'-gac gtC acT Ttg tac ggT acT att aaG gcT ggT gtT gaG act tcc cgc tct gta ttt cac cag aac ggc caa gtt act gaa gtt aca-3'.

10. The method according to claim 8, wherein said yeast is *P. pastoris*.

11. The method of claim 1 wherein said yeast secretes said protein or fusion protein into a growth medium.

12. The method of claim 11 wherein said plasmid is selected from the group consisting of pHIL-S1, pPIC9, and pPIC9K.

13. A method of purifying the outer membrane meningococcal group B porin protein or fusion protein thereof obtained according to the method of claim 1 comprising:

(a) lysing said yeast obtained in step (d) to release said protein or fusion protein as an insoluble membrane bound fraction;

(b) washing said insoluble membrane bound fraction obtained in step (a) with a buffer to remove contaminating yeast cellular proteins;

(c) suspending and dissolving said insoluble membrane bound fraction obtained in step (b) in an aqueous solution of a denaturant;

(d) diluting the solution obtained in step (c) with a detergent; and

(e) purifying said protein or fusion protein by gel filtration and ion exchange chromatography.

14. A method of purifying the outer membrane meningococcal group B porin protein or fusion protein thereof obtained according to the method of claim 11 comprising:

(a) centrifuging said yeast culture which has expressed the protein to isolate the protein as soluble secreted material;

(b) removing contaminating yeast culture impurities from the soluble secreted material obtained in step (a) by precipitating said impurities with about 20% ethanol, wherein the soluble secreted material remains in the soluble fraction;

(c) precipitating the secreted material from the soluble fraction of step (b) with about 80% ethanol;

(d) washing the precipitated material obtained in step (c) with a buffer to remove contaminating yeast secreted proteins;

(e) suspending and dissolving the precipitated material obtained in step (d) in an aqueous solution of detergent; and

(f) purifying the protein by ion exchange chromatography.

15. A yeast host cell that contains a gene coding for a protein selected from the group consisting of:

(a) a mature porin protein

(b) a fusion protein which is a mature porin protein fused to a yeast secretion signal peptide.

16. The yeast host cell of claim 15, wherein said yeast contains more than one copy of said gene.

17. The yeast host cell of claim 15 wherein said mature porin protein is the *Neisseria meningitidis* mature outer membrane class 3 protein from serogroup B.

18. The yeast host cell of claim 17 wherein said plasmid is selected from the group consisting of pHIL-D2, pHIL-S1, pPIC9, pPIC9K and pAO815.

19. The yeast host cell of claim 15, wherein said yeast is *P. pastoris.*

20. The yeast host cell of claim 15, wherein the 5' region of the gene encoding said protein is encoded by the nucleotide sequence:

5'-gac gtC acT Ttg tac ggT acT att aaG gcT ggT gtT gaG act tcc cgc tct gta ttt cac cag aac ggc caa gtt act gaa gtt aca-3'.

21. A nucleotide sequence coding for an outer membrane meningococcal group B porin protein, wherein at least one codon has been changed to optimize yeast codon usage.

22. The nucleotide sequence of claim 21, wherein said porin protein is the mature outer membrane class 3 protein from serogroup B, and said codon changes are selected from the group of changes consisting of: (GTT to GTC at positions 4-6 of the native sequence), (ACC to ACT at positions 7-9 of the native sequence), (CTG to TTG at positions 10-12 of the native sequence), (GGC to GGT at positions 16-18 of the native sequence), (ACC to ACT at positions 19-21 of the native sequence), (ATC to ATT at positions 22-24 of the native sequence), (AAA to AAG at positions 25-27 of the native sequence), (GCC to GCT at positions 28-30 of the native sequence), (GGC to GGT at positions 31-33 of the native sequence), (GTA to GTT at positions 34-36 of the native sequence), (GAA to GAG at positions 37-39 of the native sequence);
wherein said positions are numbered from the first nucleotide of the native nucleotide sequence encoding said protein.

23. A vaccine comprising group A meningococcal polysaccharide (GAMP), group B meningococcal polysaccharide (GBMP), and group C meningococcal polysaccharide (GCMP) antigens, together with a pharmaceutically acceptable carrier.

24. The vaccine of claim 23, wherein said group A meningococcal polysaccharide (GAMP), group B meningococcal polysaccharide (GBMP), and

group C meningococcal polysaccharide (GCMP) antigens are each conjugated to a protein carrier.

25. The vaccine of claim 24, wherein said protein carrier to which said GBMP antigen is conjugated is class 3 *N. meningitidis* porin protein (PorB).

26. The vaccine of claim 24, wherein said protein carrier to which said GAMP antigen and said GCMP antigen are conjugated is tetanus toxoid.

27. The vaccine of claim 25, wherein said GBMP antigen is N-propionylated prior to being conjugated to PorB.

28. The vaccine of claim 24 wherein said vaccine comprises about 2 μg of the GAMP, GCMP and GBMP polysaccharide antigen conjugates.

29. The vaccine of claim 24, wherein said vaccine comprises about 5 μg of the GAMP, GCMP and GBMP polysaccharide antigen conjugates.

30. The vaccine of claim 24, wherein said vaccine comprises about 2 μg of the GAMP and GCMP polysaccharide antigen conjugates, and about 5 μg of the GBMP polysaccharide antigen conjugate.

31. A method of inducing an immune response in a mammal, comprising administering a vaccine comprising group A meningococcal polysaccharide (GAMP), group B meningococcal polysaccharide (GBMP), and group C meningococcal polysaccharide (GCMP) antigens, together with a pharmaceutically acceptable carrier, in an amount sufficient to induce an immune response in a mammal.

32. The method of claim 31, wherein said group A meningococcal polysaccharide (GAMP), group B meningococcal polysaccharide (GBMP), and group C meningococcal polysaccharide (GCMP) antigens are each conjugated to a protein carrier.

33. The method of claim 31, wherein said mammal is a human.

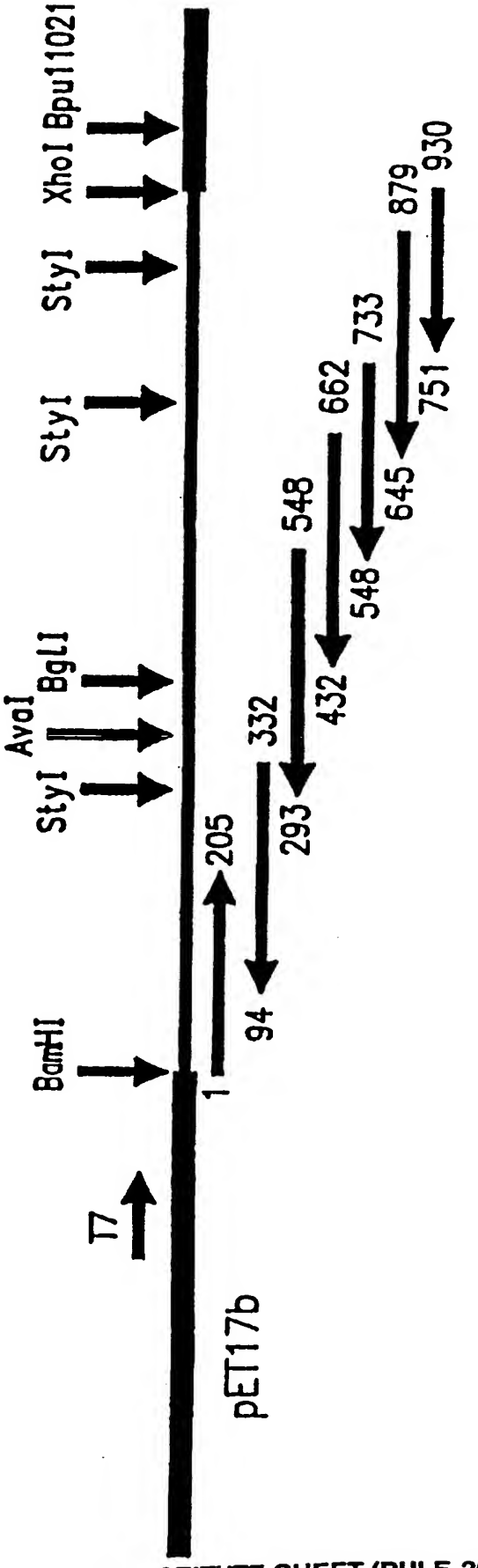

FIG.1

FIG.2

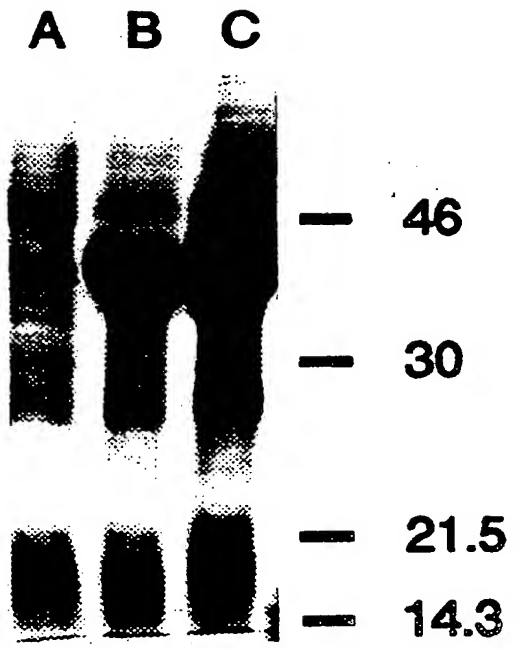

FIG.3A

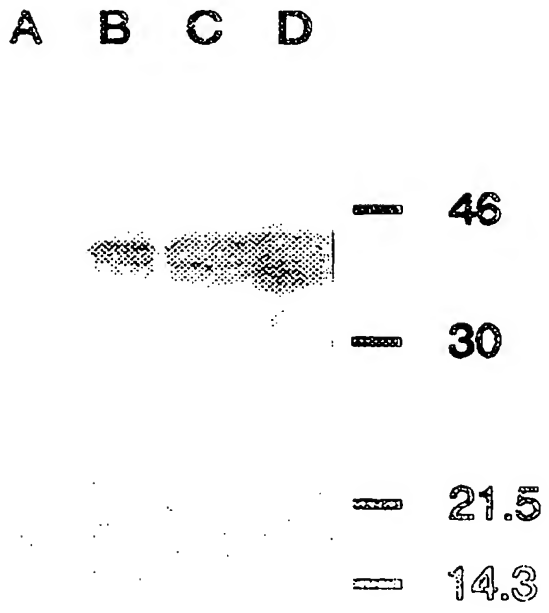

FIG.3B

```
        10 C C     20        30        40        50        60        70
TTGTACGGTACAATTAAAGCAGGCGTAGAAACTTCCCGCTCTGTATTTCACCAGAACGGCCAAGTTACTG
AACATGCCATGTTAATTTCGTCCGCATCTTTGAAGGGCGAGACATAAAGTGGTCTTGCCGGTTCAATGAC
 L  Y  G  T  I  K  A  G  V  E  T  S  R  S  V  F  H  Q  N  G  Q  V  T

        80        90       100       110       120       130       140
AAGTTACAACCGCTACCGGCATCGTTGATTTGGGTTCGAAAATCGGCTTCAAAGGCCAAGAAGACCTCGG
TTCAATGTTGGCGATGGCCGTAGCAACTAAACCCAAGCTTTTAGCCGAAGTTTCCGGTTCTTCTGGAGCC
E  V  T  T  A  T  G  I  V  D  L  G  S  K  I  G  F  K  G  Q  E  D  L  G

       150       160       170       180       190       200       210
TAACGGCCTGAAAGCCATTTGGCAGGTTGAGCAAAAAGCATCTATCGCCGGTACTGACTCCGGTTGGGGC
ATTGCCGGACTTTCGGTAAACCGTCCAACTCGTTTTTCGTAGATAGCGGCCATGACTGAGGCCAACCCCG
  N  G  L  K  A  I  W  Q  V  E  Q  K  A  S  I  A  G  T  D  S  G  W  G

       220       230       240       250       260       270       280
AACCGCCAATCCTTCATCGGCTTGAAAGGCGGCTTCGGTAAATTGCGCGTCGGTCGTTTGAACAGCGTCC
TTGGCGGTTAGGAAGTAGCCGAACTTTCCGCCGAAGCCATTTAACGCGCAGCCAGCAAACTTGTCGCAGG
 N  R  Q  S  F  I  G  L  K  G  G  F  G  K  L  R  V  G  R  L  N  S  V

       290       300       310       320       330       340       350
TGAAAGACACCGGCGACATCAATCCTTGGGATAGCAAAAGCGACTATTTGGGTGTAAACAAAATTGCCGA
ACTTTCTGTGGCCGCTGTAGTTAGGAACCCTATCGTTTTCGCTGATAAACCCACATTTGTTTTAACGGCT
L  K  D  T  G  D  I  N  P  W  D  S  K  S  D  Y  L  G  V  N  K  I  A  E

       360       370       380       390       400       410       420
ACCCGAGGCACGCCTCATTTCCGTACGCTACGATTCTCCCGAATTTGCCGGCCTCAGCGGCAGCGTACAA
TGGGCTCCGTGCGGAGTAAAGGCATGCGATGCTAAGAGGGCTTAAACGGCCGGAGTCGCCGTCGCATGTT
  P  E  A  R  L  I  S  V  R  Y  D  S  P  E  F  A  G  L  S  G  S  V  Q

       430       440       450       460       470       480       490
TACGCGCTTAACGACAATGCAGGCAGACATAACAGCGAATCTTACCACGCCGGCTTCAACTACAAAAACG
ATGCGCGAATTGCTGTTACGTCCGTCTGTATTGTCGCTTAGAATGGTGCGGCCGAAGTTGATGTTTTTGC
 Y  A  L  N  D  N  A  G  R  H  N  S  E  S  Y  H  A  G  F  N  Y  K  N

       500       510       520       530       540       550       560
GTGGCTTCTTCGTGCAATATGGCGGTGCCTATAAAAGACATCATCAAGTGCAAGAGGGCTTGAATATTGA
CACCGAAGAAGCACGTTATACCGCCACGGATATTTTCTGTAGTAGTTCACGTTCTCCCGAACTTATAACT
G  G  F  F  V  Q  Y  G  G  A  Y  K  R  H  H  Q  V  Q  E  G  L  N  I  E

       570       580       590       600       610       620       630
GAAATACCAGATTCACCGTTTGGTCAGCGGTTACGACAATGATGCCCTGTACGCTTCCGTAGCCGTACAG
CTTTATGGTCTAAGTGGCAAACCAGTCGCCAATGCTGTTACTACGGGACATGCGAAGGCATCGGCATGTC
  K  Y  Q  I  H  R  L  V  S  G  Y  D  N  D  A  L  Y  A  S  V  A  V  Q

       640       650       660       670       680       690       700
CAACAAGACGCGAAACTGACTGATGCTTCCAATTCGCACAACTCTCAAACCGAAGTTGCCGCTACCTTGG
GTTGTTCTGCGCTTTGACTGACTACGAAGGTTAAGCGTGTTGAGAGTTTGGCTTCAACGGCGATGGAACC
 Q  Q  D  A  K  L  T  D  A  S  N  S  H  N  S  Q  T  E  V  A  A  T  L
```

FIG.4A

```
       710        720        730        740        750        760        770
CATACCGCTTCGGCAACGTAACGCCCCGAGTTTCTTACGCCCACGGCTTCAAAGGTTTGGTTGATGATGC
GTATGGCGAAGCCGTTGCATTGCGGGGCTCAAAGAATGCGGGTGCCGAAGTTTCCAAACCAACTACTACG
A  Y  R  F  G  N  V  T  P  R  V  S  Y  A  H  G  F  K  G  L  V  D  D  A

       780        790        800        810        820        830        840
AGACATAGGCAACGAATACGACCAAGTGGTTGTCGGTGCGGAATACGACTTCTCCAAACGCACTTCTGCC
TCTGTATCCGTTGCTTATGCTGGTTCACCAACAGCCACGCCTTATGCTGAAGAGGTTTGCGTGAAGACGG
  D  I  G  N  E  Y  D  Q  V  V  V  G  A  E  Y  D  F  S  K  R  T  S  A

       850        860        870        880        890        900        910
TTGGTTTCTGCCGGTTGGTTGCAAGAAGGCAAAGGCGAAAACAAATTCGTAGCGACTGCCGGCGGTGTTG
AACCAAAGACGGCCAACCAACGTTCTTCCGTTTCCGCTTTTGTTTAAGCATCGCTGACGGCCGCCACAAC
 L  V  S  A  G  W  L  Q  E  G  K  G  E  N  K  F  V  A  T  A  G  G  V

       920        930
GTCTGCGTCACAAATTCTAA
CAGACGCAGTGTTTAAGATT
G  L  R  H  K  F
```

FIG.4A-1

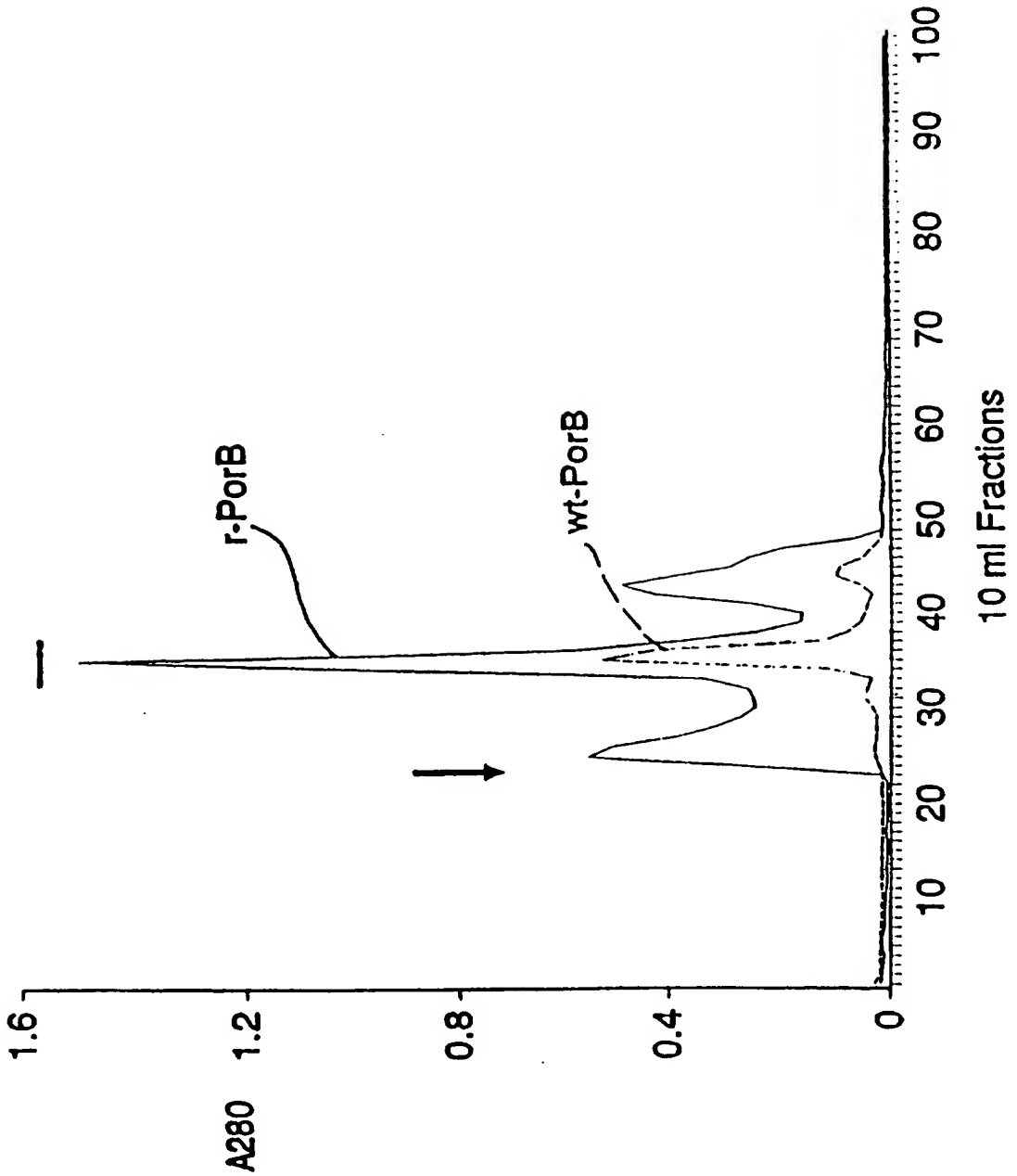

FIG.5

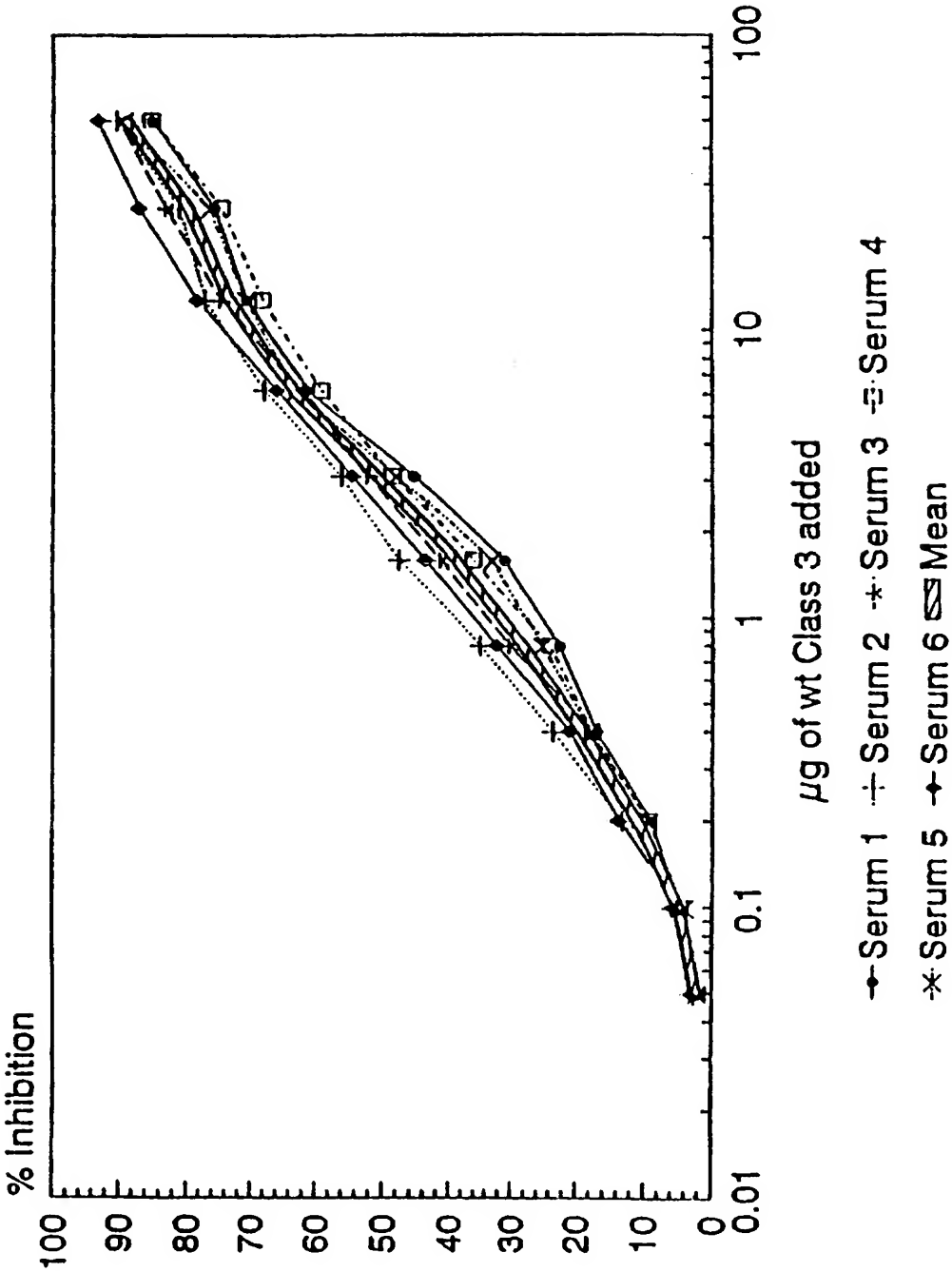

FIG.6

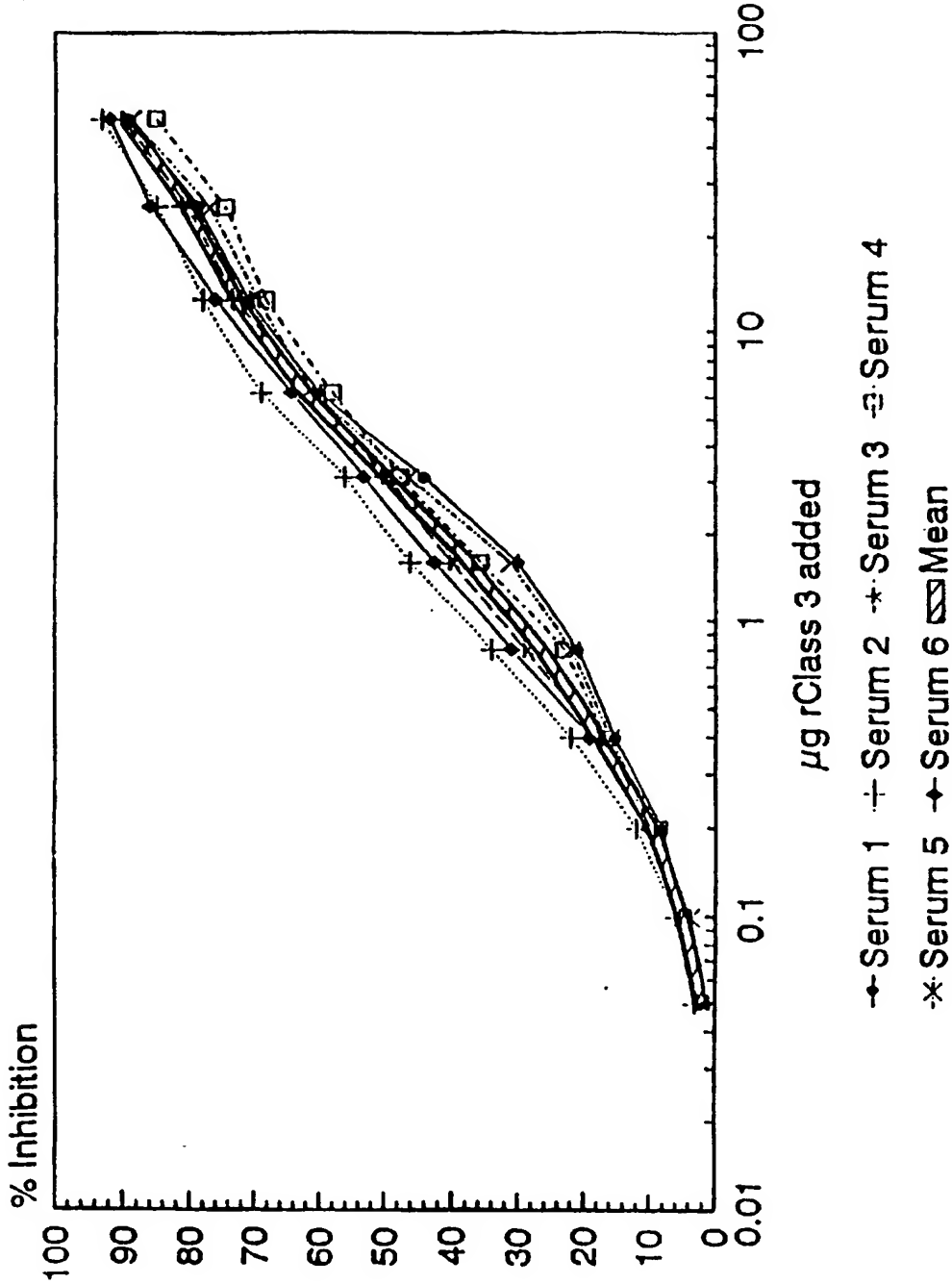

FIG. 7

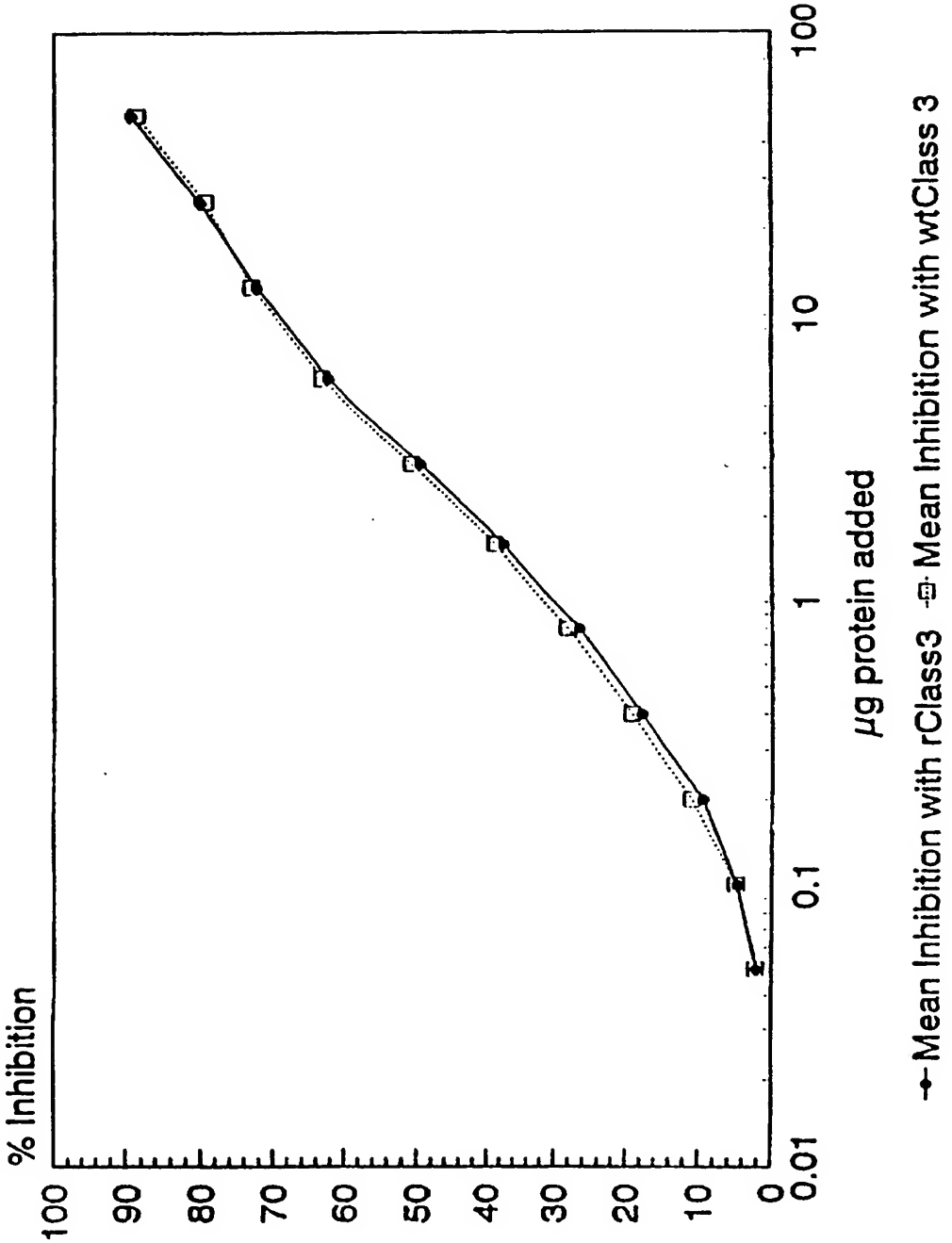

FIG. 8

```
ATGGACGTTACCTTGTACGGTACAATTAAAGCAGGCGTAGAAGTTTCTCGCGTAAAAGATGCTGGTACAT 70
TACCTGCAATGGAACATGCCATGTTAATTTCGTCCGCATCTTCAAAGAGCGCATTTTCTACGACCATGTA
 M  D  V  T  L  Y  G  T  I  K  A  G  V  E  V  S  R  V  K  D  A  G  T

ATAAAGCTCAAGGCGGAAAATCTAAAACTGCAACCCAAATTGCCGACTTCGGTTCTAAAATCGGTTTCAA 140
TATTTCGAGTTCCGCCTTTTAGATTTTGACGTTGGGTTTAACGGCTGAAGCCAAGATTTTAGCCAAAGTT
 Y  K  A  Q  G  G  K  S  K  T  A  T  Q  I  A  D  F  G  S  K  I  G  F  K

AGGTCAAGAAGACCTCGGCAACGGCATGAAAGCCATTTGGCAGTTGGAACAAAAAGCCTCCATCGCCGGC 210
TCCAGTTCTTCTGGAGCCGTTGCCGTACTTTCGGTAAACCGTCAACCTTGTTTTTCGGAGGTAGCGGCCG
  G  Q  E  D  L  G  N  G  M  K  A  I  W  Q  L  E  Q  K  A  S  I  A  G

ACTAACAGCGGCTGGGGTAACCGCCAGTCCTTCATCGGCTTGAAAGGCGGCTTCGGTACCGTCCGCGCCG 280
TGATTGTCGCCGACCCCATTGGCGGTCAGGAAGTAGCCGAACTTTCCGCCGAAGCCATGGCAGGCGCGGC
 T  N  S  G  W  G  N  R  Q  S  F  I  G  L  K  G  G  F  G  T  V  R  A

GTAATCTGAACACCGTATTGAAAGACAGCGGCGACAACGTCAATGCATGGGAATCTGGTTCTAACACCGA 350
CATTAGACTTGTGGCATAACTTTCTGTCGCCGCTGTTGCAGTTACGTACCCTTAGACCAAGATTGTGGCT
G  N  L  N  T  V  L  K  D  S  G  D  N  V  N  A  W  E  S  G  S  N  T  E

AGATGTACTGGGACTGGGTACTATCGGTCGTGTAGAAAGCCGTGAAATCTCCGTACGCTACGACTCTCCC 420
TCTACATGACCCTGACCCATGATAGCCAGCACATCTTTCGGCACTTTAGAGGCATGCGATGCTGAGAGGG
  D  V  L  G  L  G  T  I  G  R  V  E  S  R  E  I  S  V  R  Y  D  S  P

GTATTTGCAGGCTTCAGCGGCAGCGTACAATACGTTCCGCGCGATAATGCGAATGATGTGGATAAATACA 490
CATAAACGTCCGAAGTCGCCGTCGCATGTTATGCAAGGCGCGCTATTACGCTTACTACACCTATTTATGT
 V  F  A  G  F  S  G  S  V  Q  Y  V  P  R  D  N  A  N  D  V  D  K  Y

AACATACGAAGTCCAGCCGTGAGTCTTACCACGCCGGTCTGAAATACGAAAATGCCGGTTTCTTCGGTCA 560
TTGTATGCTTCAGGTCGGCACTCAGAATGGTGCGGCCAGACTTTATGCTTTTACGGCCAAAGAAGCCAGT
K  H  T  K  S  S  R  E  S  Y  H  A  G  L  K  Y  E  N  A  G  F  F  G  Q

ATACGCAGGTTCTTTTGCCAAATATGCTGATTTGAACACTGATGCAGAACGTGTTGCAGTAAATACTGCA 630
TATGCGTCCAAGAAAACGGTTTATACGACTAAACTTGTGACTACGTCTTGCACAACGTCATTTATGACGT
  Y  A  G  S  F  A  K  Y  A  D  L  N  T  D  A  E  R  V  A  V  N  T  A
```

FIG.9A

```
AATGCCCATCCTGTTAAGGATTACCAAGTACACCGCGTAGTTGCCGGTTACGATGCCAATGACCTGTACG 700
TTACGGGTAGGACAATTCCTAATGGTTCATGTGGCGCATCAACGGCCAATGCTACGGTTACTGGACATGC
 N  A  H  P  V  K  D  Y  Q  V  H  R  V  V  A  G  Y  D  A  N  D  L  Y

TTTCTGTTGCCGGTCAGTATGAAGCTGCTAAAAACAACGAGGTTGGTTCTACCAAGGGTAAAAAACACGA 770
AAAGACAACGGCCAGTCATACTTCGACGATTTTTGTTGCTCCAACCAAGATGGTTCCCATTTTTTGTGCT
V  S  V  A  G  Q  Y  E  A  A  K  N  N  E  V  G  S  T  K  G  K  K  H  E

GCAAACTCAAGTTGCCGCTACTGCCGCTTACCGTTTTGGCAACGTAACGCCTCGCGTTTCTTACGCCCAC 840
CGTTTGAGTTCAACGGCGATGACGGCGAATGGCAAAACCGTTGCATTGCGGAGCGCAAAGAATGCGGGTG
  Q  T  Q  V  A  A  T  A  A  Y  R  F  G  N  V  T  P  R  V  S  Y  A  H
```

FIG.9A-1

```
GGCTTCAAAGCTAAAGTGAATGGCGTGAAAGACGCAAATTACCAATACGACCAAGTTATCGTTGGTGCCG 910
CCGAAGTTTCGATTTCACTTACCGCACTTTCTGCGTTTAATGGTTATGCTGGTTCAATAGCAACCACGGC
 G  F  K  A  K  V  N  G  V  K  D  A  N  Y  Q  Y  D  Q  V  I  V  G  A

ACTACGACTTCTCCAAACGCACTTCCGCTCTGGTTTCTGCCGGTTGGTTGAAACAAGGTAAAGGCGCGGG 980
TGATGCTGAAGAGGTTTGCGTGAAGGCGAGACCAAAGACGGCCAACCAACTTTGTTCCATTTCCGCGCCC
D  Y  D  F  S  K  R  T  S  A  L  V  S  A  G  W  L  K  Q  G  K  G  A  G

AAAAGTCGAACAAACTGCCAGCATGGTTGGTCTGCGTCACAAATTCTAA 1029
TTTTCAGCTTGTTTGACGGTCGTACCAACCAGACGCAGTGTTTAAGATT
  K  V  E  Q  T  A  S  M  V  G  L  R  H  K  F
```

FIG.9B

```
                                                                    70
ATGGCTAGCATGACTGGTGGACAGCAAATGGGTCGGGATTCAAGCTTGGTACCGAGCTCGGATCCAGACG
TACCGATCGTACTGACCACCTGTCGTTTACCCAGCCCTAAGTTCGAACCATGGCTGGAGCCTAGGTCTGC
  M  A  S  M  T  G  G  Q  Q  M  G  R  D  S  S  L  V  P  S  S  D  P  D

                                                                   140
TTACCTTGTACGGTACAATTAAAGCAGGCGTAGAAGTTTCTCGCGTAAAAGATGCTGGTACATATAAAGC
AATGGAACATGCCATGTTAATTTCGTCCGCATCTTCAAAGAGCGCATTTTCTACGACCATGTATATTTCG
 V  T  L  Y  G  T  I  K  A  G  V  E  V  S  R  V  K  D  A  G  T  Y  K  A

                                                                   210
TCAAGGCGGAAAATCTAAAACTGCAACCCAAATTGCCGACTTCGGTTCTAAAATCGGTTTCAAAGGTCAA
AGTTCCGCCTTTTAGATTTTGACGTTGGGTTTAACGGCTGAAGCCAAGATTTTAGCCAAAGTTTCCAGTT
  Q  G  G  K  S  K  T  A  T  Q  I  A  D  F  G  S  K  I  G  F  K  G  Q

                                                                   280
GAAGACCTCGGCAACGGCATGAAAGCCATTTGGCAGTTGGAACAAAAAGCCTCCATCGCCGGCACTAACA
CTTCTGGAGCCGTTGCCGTACTTTCGGTAAACCGTCAACCTTGTTTTTCGGAGGTAGCGGCCGTGATTGT
 E  D  L  G  N  G  M  K  A  I  W  Q  L  E  Q  K  A  S  I  A  G  T  N

                                                                   350
GCGGCTGGGGTAACCGCCAGTCCTTCATCGGCTTGAAAGGCGGCTTCGGTACCGTCCGCGCCGGTAATCT
CGCCGACCCCATTGGCGGTCAGGAAGTAGCCGAACTTTCCGCCGAAGCCATGGCAGGCGCGGCCATTAGA
 S  G  W  G  N  R  Q  S  F  I  G  L  K  G  G  F  G  T  V  R  A  G  N  L

                                                                   420
GAACACCGTATTGAAAGACAGCGGCGACAACGTCAATGCATGGGAATCTGGTTCTAACACCGAAGATGTA
CTTGTGGCATAACTTTCTGTCGCCGCTGTTGCAGTTACGTACCCTTAGACCAAGATTGTGGCTTCTACAT
  N  T  V  L  K  D  S  G  D  N  V  N  A  W  E  S  G  S  N  T  E  D  V

                                                                   490
CTGGGACTGGGTACTATCGGTCGTGTAGAAAGCCGTGAAATCTCCGTACGCTACGACTCTCCCGTATTTG
GACCCTGACCCATGATAGCCAGCACATCTTTCGGCACTTTAGAGGCATGCGATGCTGAGAGGGCATAAAC
 L  G  L  G  T  I  G  R  V  E  S  R  E  I  S  V  R  Y  D  S  P  V  F

                                                                   560
CAGGCTTCAGCGGCAGCGTACAATACGTTCCGCGCGATAATGCGAATGATGTGGATAAATACAAACATAC
GTCCGAAGTCGCCGTCGCATGTTATGCAAGGCGCGCTATTACGCTTACTACACCTATTTATGTTTGTATG
 A  G  F  S  G  S  V  Q  Y  V  P  R  D  N  A  N  D  V  D  K  Y  K  H  T
```

FIG. 10A

630

GAAGTCCAGCCGTGAGTCTTACCACGCCGGTCTGAAATACGAAAATGCCGGTTTCTTCGGTCAATACGCA
CTTCAGGTCGGCACTCAGAATGGTGCGGCCAGACTTTATGCTTTTACGGCCAAAGAAGCCAGTTATGCGT
K S S R E S Y H A G L K Y E N A G F F G Q Y A

700

GGTTCTTTTGCCAAATATGCTGATTTGAACACTGATGCAGAACGTGTTGCAGTAAATACTGCAAATGCCC
CCAAGAAAACGGTTTATACGACTAAACTTGTGACTACGTCTTGCACAACGTCATTTATGACGTTTACGGG
G S F A K Y A D L N T D A E R V A V N T A N A

FIG.10A-1

770

ATCCTGTTAAGGATTACCAAGTACACCGCGTAGTTGCCGGTTACGATGCCAATGACCTGTACGTTTCTGT
TAGGACAATTCCTAATGGTTCATGTGGCGCATCAACGGCCAATGCTACGGTTACTGGACATGCAAAGACA
H P V K D Y Q V H R V V A G Y D A N D L Y V S V

840

TGCCGGTCAGTATGAAGCTGCTAAAAACAACGAGGTTGGTTCTACCAAGGGTAAAAAACACGAGCAAACT
ACGGCCAGTCATACTTCGACGATTTTTGTTGCTCCAACCAAGATGGTTCCCATTTTTTGTGCTCGTTTGA
A G Q Y E A A K N N E V G S T K G K K H E Q T

910

CAAGTTGCCGCTACTGCCGCTTACCGTTTTGGCAACGTAACGCCTCGCGTTTCTTACGCCCACGGCTTCA
GTTCAACGGCGATGACGGCGAATGGCAAAACCGTTGCATTGCGGAGCGCAAAGAATGCGGGTGCCGAAGT
Q V A A T A A Y R F G N V T P R V S Y A H G F

980

AAGCTAAAGTGAATGGCGTGAAAGACGCAAATTACCAATACGACCAAGTTATCGTTGGTGCCGACTACGA
TTCGATTTCACTTACCGCACTTTCTGCGTTTAATGGTTATGCTGGTTCAATAGCAACCACGGCTGATGCT
K A K V N G V K D A N Y Q Y D Q V I V G A D Y D

1050

CTTCTCCAAACGCACTTCCGCTCTGGTTTCTGCCGGTTGGTTGAAACAAGGTAAAGGCGCGGGAAAAGTC
GAAGAGGTTTGCGTGAAGGCGAGACCAAAGACGGCCAACCAACTTTGTTCCATTTCCGCGCCCTTTTCAG
F S K R T S A L V S A G W L K Q G K G A G K V

1092

GAACAAACTGCCAGCATGGTTGGTCTGCGTCACAAATTCTAA
CTTGTTTGACGGTCGTACCAACCAGACGCAGTGTTTAAGATT
E Q T A S M V G L R H K F

FIG.10B

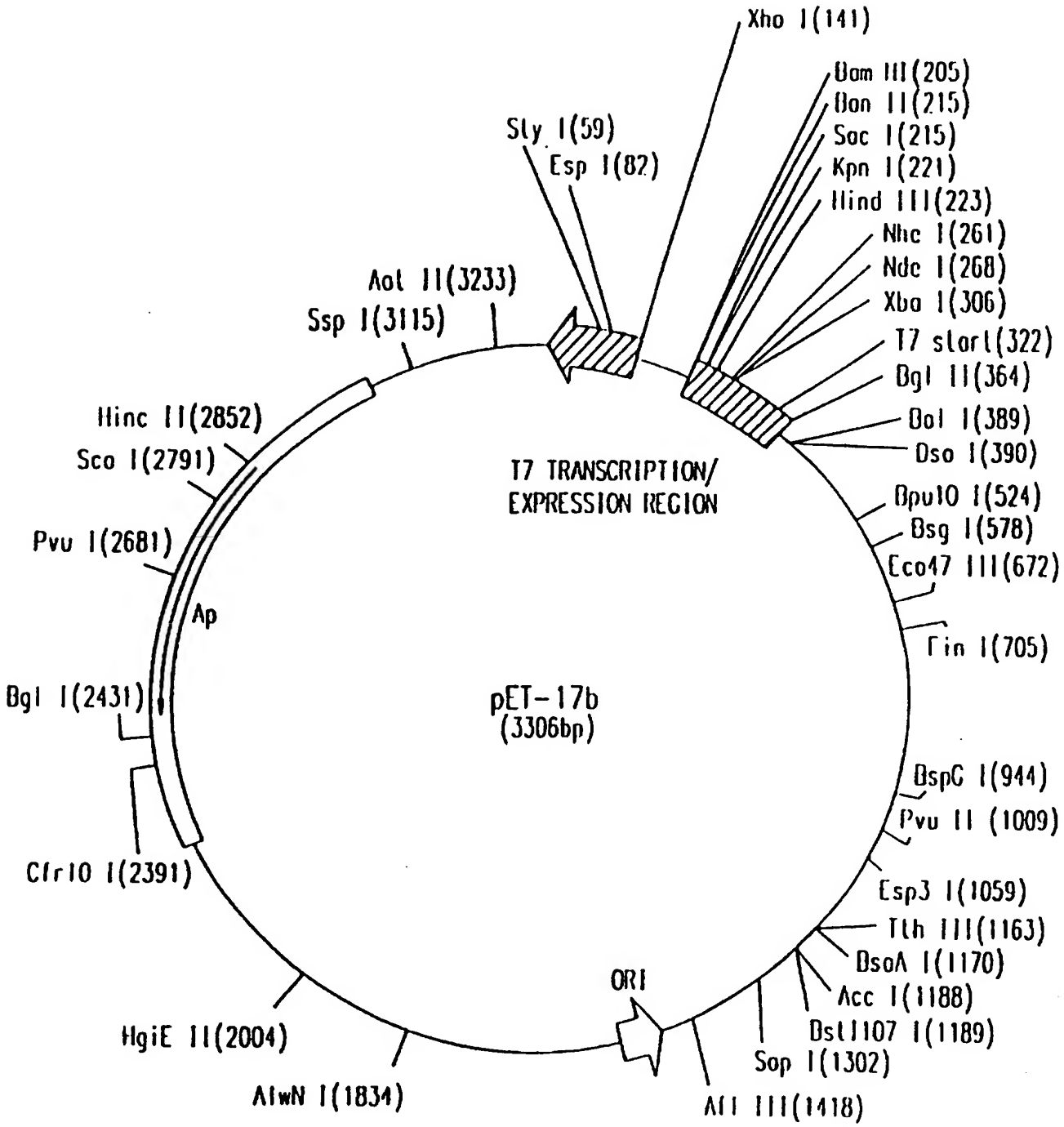

FIG.11A

```
 SgfI                                                                  XbaI
AGATCTCGAT CCCGCGAAAT TAATACGACT CACTATAGGG AGACCACAAC GGTTTCCCTC      60

                                          NdeI    NheI
TAGAAATAAT TTTGTTTAAC TTAAGAAGGA GATATACAT ATG GCT AGC ATG ACT         115
                                           Met Ala Ser Met Thr
                                            1               5

                                    HindIII KpnI   SacI   (BamHI)
GGT CGA CAG CAA ATG GGT CGG GAT TCA AGC TTG GTA CCG AGC TCG GAT        163
Gly Gly Gln Gln Met Gly Arg Asp Ser Ser Leu Val Pro Ser Ser Asp
                10                  15                  20

                ---------->
CTG CAG GTT ACC TTG TAC GGT ACA                                        187
Leu Gln Val Thr Leu Tyr Gly Thr
            25

<----------------------------- XbaI
GTT GGT CTG CGT CAC AAA TTC TAACTCGAGC AGATCCGGCT GCTAACAAAG           51
Val Gly Leu Arg His Lys Phe
 1              5

CCC                                                                    54
```

FIG.11B

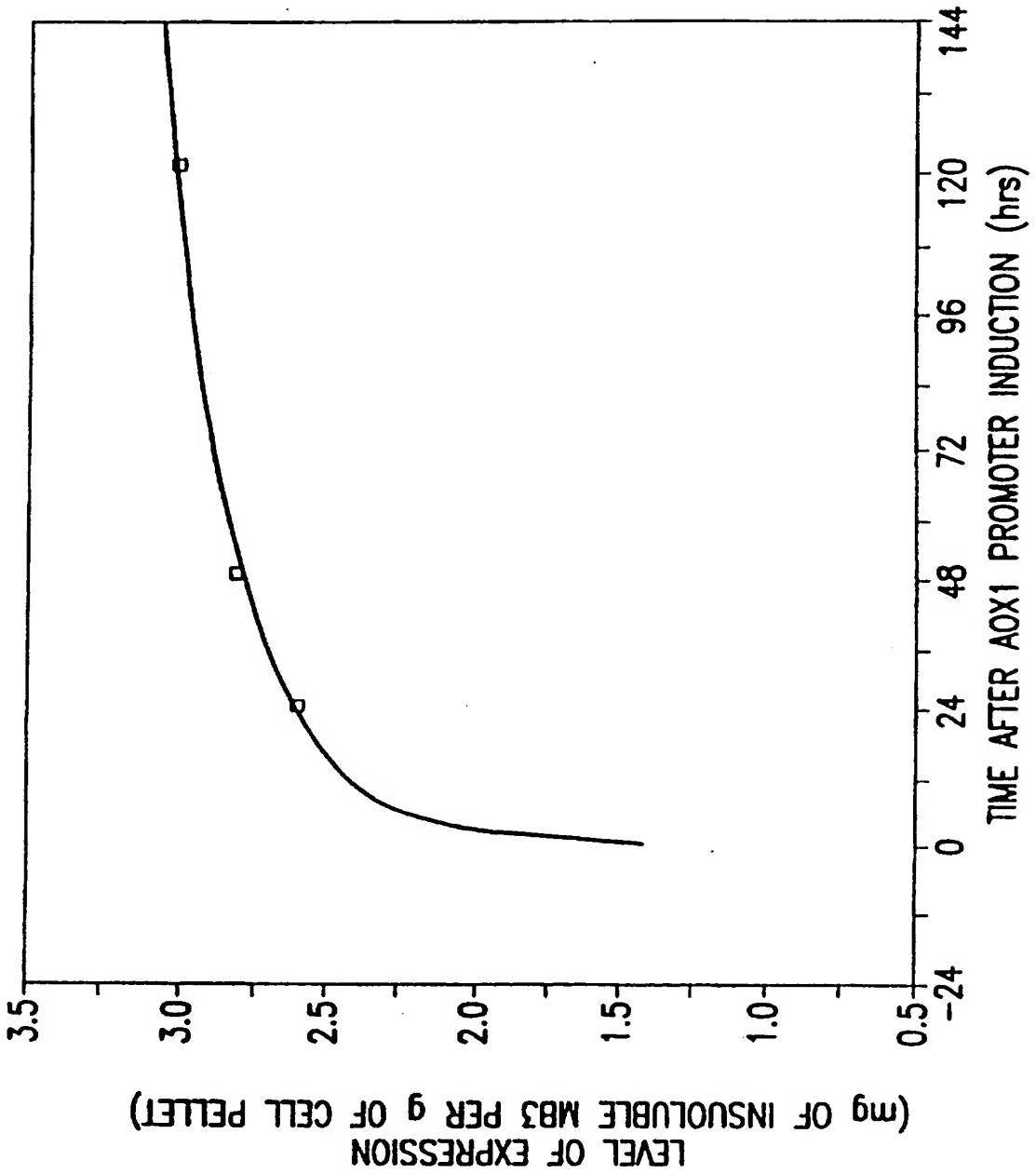

FIG.12

```
1   /   1                                   31   /  11
ATG gac gtt acc ctg tac ggc acc att aaa gca ggc gta gaa act tcc cgc tct gta ttt
met asp val thr leu tyr gly thr ile lys ala gly val glu thr ser arg ser val phe

61   /  21                                  91   /  31
cac cag aac ggc caa gtt act gaa gtt aca acc gct acc ggc atc gtt gat ttg ggt gcg
his gln asn gly gln val thr glu val thr thr ala thr gly ile val asp leu gly ser

121  /  41                                  151  /  51
aaa atc ggc ttc aaa ggc caa gaa gac ctc ggt aac ggc ctg aaa gcc att tgg cag gtt
lys ile gly phe lys gly gln glu asp leu gly asn gly leu lys ala ile trp gln val

181  /  61                                  211  /  71
gag caa aaa gca tct atc gcc ggt act gac tcc ggt tgg ggc aac cgc caa tcc ttc atc
glu gln lys ala ser ile ala gly thr asp ser gly trp gly asn arg gln ser phe ile

241  /  81                                  271  /  91
ggc ttg aaa ggc ggc ttc ggt aaa ttg cgc gtc ggt cgt ttg aac agc gtc ctg aaa gac
gly leu lys gly gly phe gly lys leu arg val gly arg leu asn ser val leu lys asp

301  /  101                                 331  /  111
acc ggc gac atc aat cct tgg gat agc aaa agc gac tct ttg ggt gta aac aaa att gcc
thr gly asp ile asn pro trp asp ser lys ser asp tyr leu gly val asn lys ile ala

361  /  121                                 391  /  131
gaa ccc gag gca cgc gtg att tcc gta cgc tac gat tct ccc gaa ttt gcc ggc ctc agc
glu pro glu ala arg leu ile ser val arg tyr asp ser pro glu phe ala gly leu ser

421  /  141                                 451  /  151
ggc agc gta caa tac gcg ctt aac gac aat gca ggc aga cat aac agc gaa ttc tac cac
gly ser val gln tyr ala leu asn asp asn ala gly arg his asn ser glu ser tyr his

481  /  161                                 511  /  171
gcc ggc ttc aac tac aaa aac ggt ggc ttc ttc gag caa tct ggc ggt gcc tat aaa aga
ala gly phe asn tyr lys asn gly gly phe phe val gln tyr gly gly ala tyr lys arg
```

FIG. 13A

```
541  /  181                                  571  /  191
cat cat caa gtg caa gag ggc ttg aat att gag aaa tac cag att cac cgt ttg gtc agc
his his gln val gln glu gly leu asn ile glu lys tyr gln ile his arg leu val ser

601  /  201                                  631  /  211
ggt tac gac aat gat gcc ctg tac gct tcc gta gcc gta cag caa caa gac gag aaa ctg
gly tyr asp ans asp ala leu tyr ala ser val ala val gln gln gln asp ala lys leu

661  /  221                                  691  /  231
act gat gct tcc aat tcg cac aac tct caa acc gaa gtt gcc gct acc ttg gca gac cgc
thr asp ala ser asn ser his asn ser gln thr glu val ala ala thr leu ala tyr arg

721  /  241                                  751  /  251
ttc ggc aac gta acg ccc cga gtt tct tac gcc cac ggc ttc aaa ggt ttg gtt gat gat
phe gly asn val thr pro arg val ser tyr ala his gly phe lys gly leu val asp asp

781  /  261                                  811  /  271
gca gac ata ggc aac gaa tac gac caa gtg gtt gtc ggt gcg gaa tac gac ttc tcc aaa
ala asp ile gly asn glu tyr asp gln val val val gly ala glu tyr asp phe ser lys

841  /  281                                  871  /  291
cgc act tct gcc ttg gtt tct gcc ggt tgg ttg caa gaa ggc aaa ggc gaa aac aaa ttc
arg thr ser ala leu val ser ala gly trp glu gln glu gly lys gly glu asn lys phe

901  /  301                                  931  /  311
gta gcg act gcc ggc ggt gtc ggt ctg cgc cac aaa ggc taa
val ala thr ala gly gly val gly leu arg his lys phe OCH
```

FIG.13A-1

```
1   /   1                                   31   /  11
ATG gac gtC acT Ttg tac ggT acT att aaG gcT ggT gtT gaG act tcc cgc tct gta ttt
met asp val thr leu tyr gly thr ile lys ala gly val glu thr ser arg ser val phe

61   /  21                                  91   /  31
cac cag aac ggc caa gtt act gaa gtt aca acc gct acc ggc atc gtt gat ttg ggt gcg
his gln asn gly gln val thr glu val thr thr ala thr gly ile val asp leu gly ser

121  /  41                                  151  /  51
aaa atc ggc ttc aaa ggc caa gaa gac ctc ggt aac ggc ctg aaa gcc att tgg cag gtt
lys ile gly phe lys gly gln glu asp leu gly asn gly leu lys ala ile trp gln val

181  /  61                                  211  /  71
gag caa aaa gca tct atc gcc ggt act gac tcc ggt tgg ggc aac cgc caa tcc ttc atc
glu gln lys ala ser ile ala gly thr asp ser gly trp gly asn arg gln ser phe ile

241  /  81                                  271  /  91
ggc ttg aaa ggc ggc ttc ggt aaa ttg cgc gtc ggt cgt ttg aac agc gtc ctg aaa gac
gly leu lys gly gly phe gly lys leu arg val gly arg leu asn ser val leu lys asp

301  /  101                                 331  /  111
acc ggc gac atc aat cct tgg gat agc aaa agc gac tct ttg ggt gta aac aaa att gcc
thr gly asp ile asn pro trp asp ser lys ser asp tyr leu gly val asn lys ile ala

361  /  121                                 391  /  131
gaa ccc gag gca cgc gtg att tcc gta cgc tac gat tct ccc gaa ttt gcc ggc ctc agc
glu pro glu ala arg leu ile ser val arg tyr asp ser pro glu phe ala gly leu ser

421  /  141                                 451  /  151
ggc agc gta caa tac gcg ctt aac gac aat gca ggc aga cat aac agc gaa ttc tac cac
gly ser val gln tyr ala leu asn asp asn ala gly arg his asn ser glu ser tyr his

481  /  161                                 511  /  171
gcc ggc ttc aac tac aaa aac ggt ggc ttc ttc gag caa tct ggc ggt gcc tat aaa aga
ala gly phe asn tyr lys asn gly gly phe phe val gln tyr gly gly ala tyr lys arg
```

FIG.13B

541 / 181 571 / 191
cat cat caa gtg caa gag ggc ttg aat att gag aaa tac cag att cac cgt ttg gtc agc
his his gln val gln glu gly leu asn ile glu lys tyr gln ile his arg leu val ser

601 / 201 631 / 211
ggt tac gac aat gat gcc ctg tac gct tcc gta gcc gta cag caa caa gac gag aaa ctg
gly tyr asp ans asp ala leu tyr ala ser val ala val gln gln gln asp ala lys leu

661 / 221 691 / 231
act gat gct tcc aat tcg cac aac tct caa acc gaa gtt gcc gct acc ttg gca gac cgc
thr asp ala ser asn ser his asn ser gln thr glu val ala ala thr leu ala tyr arg

721 / 241 751 / 251
ttc ggc aac gta acg ccc cga gtt tct tac gcc cac ggc ttc aaa ggt ttg gtt gat gat
phe gly asn val thr pro arg val ser tyr ala his gly phe lys gly leu val asp asp

781 / 261 811 / 271
gca gac ata ggc aac gaa tac gac caa gtg gtt gtc ggt gcg gaa tac gac ttc tcc aaa
ala asp ile gly asn glu tyr asp gln val val val gly ala glu tyr asp phe ser lys

841 / 281 871 / 291
cgc act tct gcc ttg gtt tct gcc ggt tgg ttg caa gaa ggc aaa ggc gaa aac aaa ttc
arg thr ser ala leu val ser ala gly trp glu gln glu gly lys gly glu asn lys phe

901 / 301 931 / 311
gta gcg act gcc ggc ggt gtc ggt ctg cgc cac aaa ggc taa
val ala thr ala gly gly val gly leu arg his lys phe OCH

FIG.13B-1

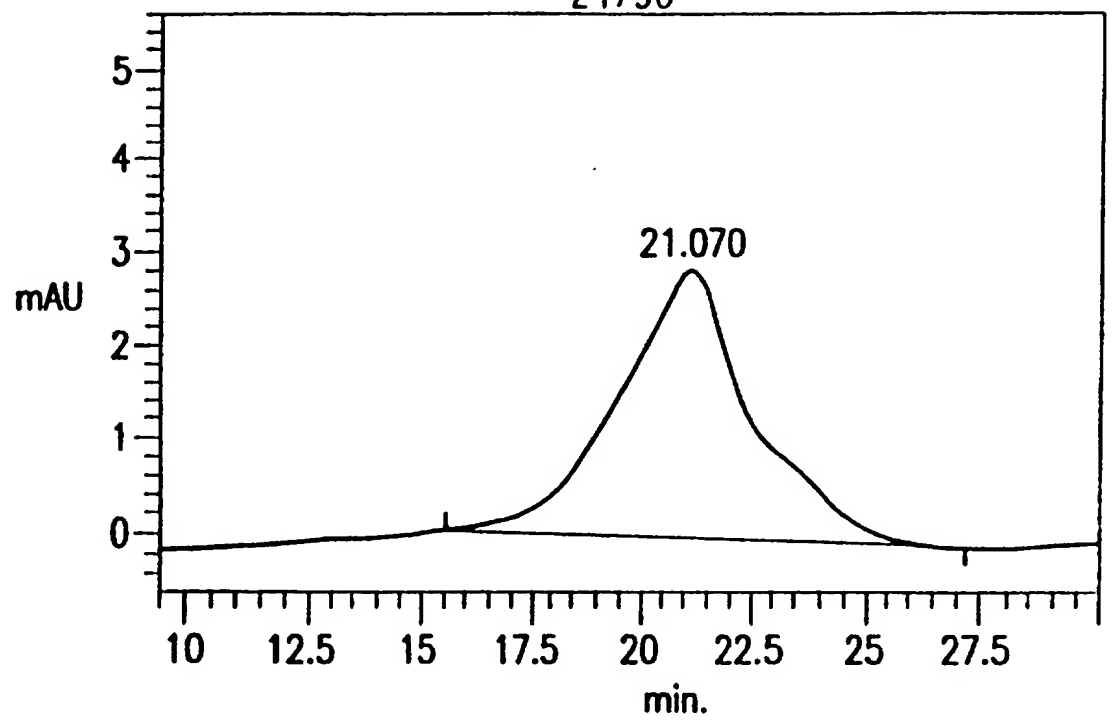

FIG.14A

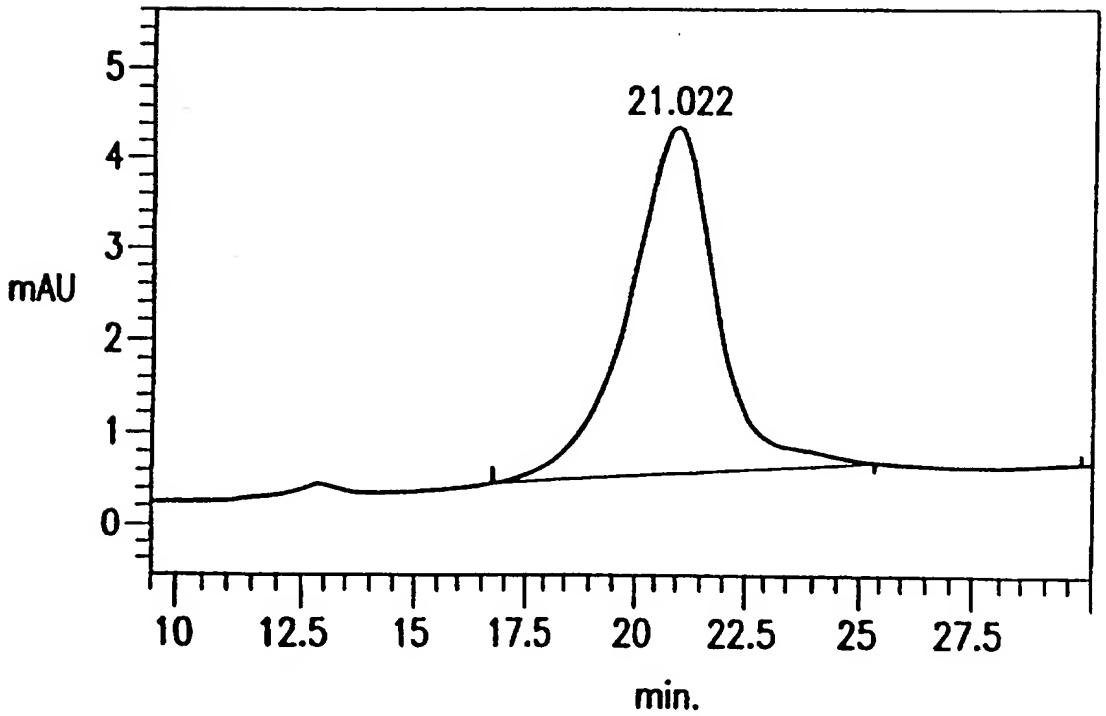

FIG.14B

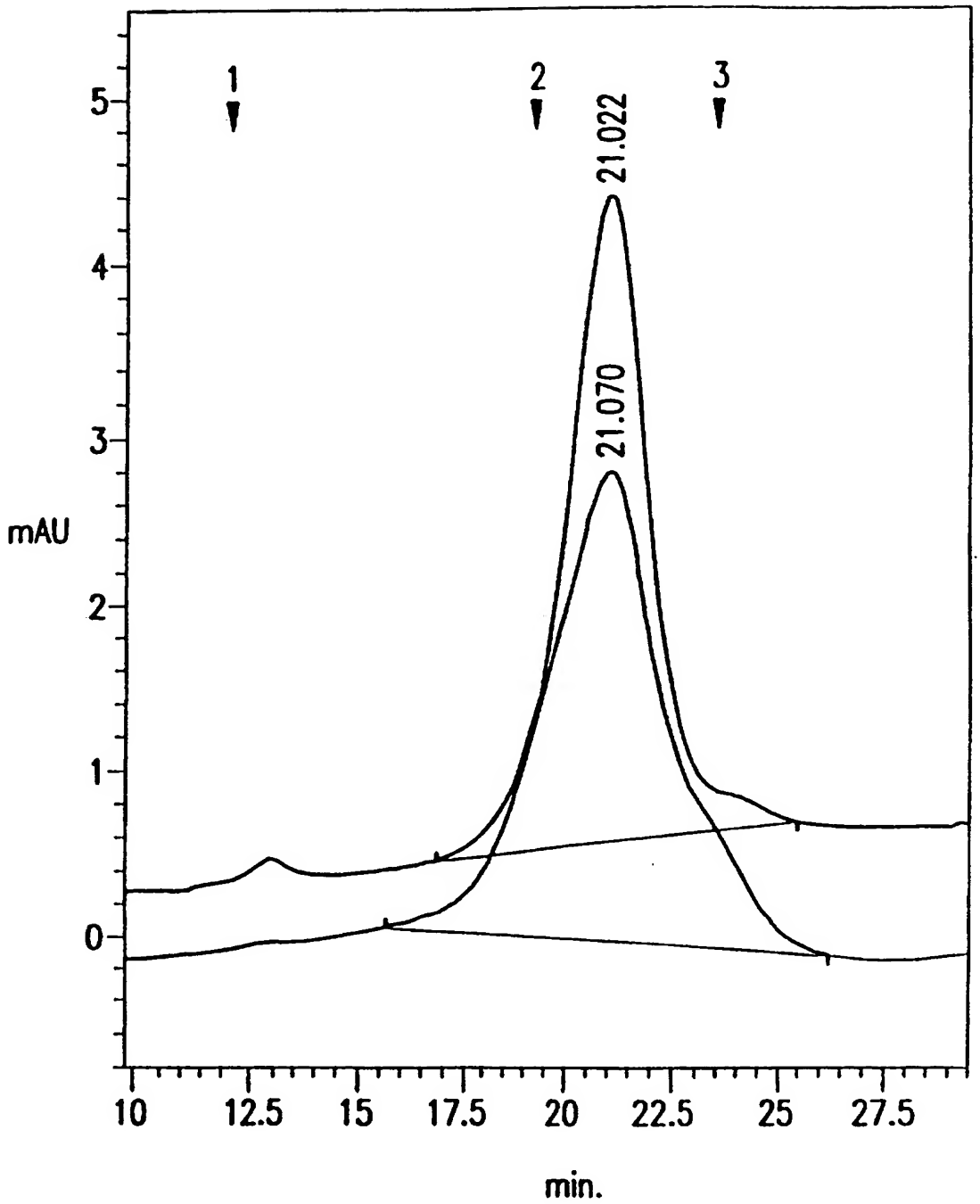

FIG.15

```
      |    10      |    20      |    30      |    40      |    50      |    60
    1 AGATCgcggc cgcgatctaa catccaaaga CGAAAGGTTG AATGAAACCT TTTTGCCATC 60
   61 CGACATCCAC AGGTCCATTC TCACACATAA GTGCCAAACG CAACAGGAGG GGATACACTA 120
  121 GCAGCAGACC GTTGCAAACG CAGGACCTCC ACTCCTCTTC TCCTCAACAC CCACTTTTGC 180
  181 CATCGAAAAA CCAGCCCAGT TATTGGGCTT GATTGGAGCT CGCTCATTCC AATTCCTTCT 240
  241 ATTAGGCTAC TAACACCATG ACTTTATTAG CCTGTCTATC CTGGCCCCCC TGGCGAGGTC 300
  301 ATGTTTGTTT ATTTCCGAAT GCAACAAGCT CCGCATTACA CCCGAACATC ACTCCAGATG 360
  361 AGGGCTTTCT GAGTGTGGGG TCAAATAGTT TCATGTTCCC AAATGGCCCA AAACTGACAG 420
  421 TTTAAACGCT GTCTTGGAAC CTAATATGAC AAAAGCGTGA TCTCATCCAA GATGAACTAA 480
  481 GTTTGGTTCG TTGAAATGCT AACGGCCAGT TGGTCAAAAA GAAACTTCCA AAAGTCGCCA 540
  541 TACCGTTTGT CTTGTTTGGT ATTGATTGAC GAATGCTCAA AAATAATCTC ATTAATGCTT 600
  601 AGCGCAGTCT CTCTATCGCT TCTGAACCCG GTGGCACCTG TGCCGAAACG CAAATGGGGA 660
  661 AACAACCCGC TTTTTGGATG ATTATGCATT GTCCTCCACA TTGTATGCTT CCAAGATTCT 720
  721 GGTGGGAATA CTGCTGATAG CCTAACGTTC ATGATCAAAA TTTAACTGTT CTAACCCCTA 780
  781 CTTGACAGGC AATATATAAA CAGAAGGAAG CTGCCCTGTC TTAAACCTTT TTTTTTATCA 840
  841 TCATTATTAG CTTACTTTCA TAATTGCGAC TGGTTCCAAT TGACAAGCTT TTGATTTTAA 900
  901 CGACTTTTAA CGACAACTTG AGAAGATCAA AAAACAACTA ATTATTCGAA ACGAGGAATT 960
  961 CATGgacgtc actttgtacg gtactattaa ggctggtgtt gagacttccc gctctgtatt 1020
 1021 tcaccagaac ggccaagtta ctgaagttac aaccgctacc ggcatcgttg atttgggttc 1080
 1081 gaaaatcggc ttcaaaggcc aagaagacct cggtaacggc ctgaaagcca tttggcaggt 1140
 1141 tgagcaaaaa gcatctatcg ccggtactga ctccggttgg ggcaaccgcc aatccttcat 1200
 1201 cggcttgaaa ggcggcttcg gtaaattgcg cgtcggtcgt ttgaacagcg tcctgaaaga 1260
 1261 caccggcgac atcaatcctt gggatagcaa aagcgactat ttgggtgtaa acaaaattgc 1320
 1321 cgaacccgag gcacgcctca tttccgtacg ctacgattct cccgaatttg ccggcctcag 1380
 1381 cggcagcgta caatacgcgc ttaacgacaa tgcaggcaga cataacagcg aatcttacca 1440
 1441 cgccggcttc aactacaaaa acggtggctt cttcgtgcaa tatggcggtg cctataaaag 1500
 1501 acatcatcaa gtgcaagagg gcttgaatat tgagaaatac cagattcacc gtttggtcag 1560
 1561 cggttacgac aatgatgccc tgtacgcttc cgtagccgta cagcaacaag acgcgaaact 1620
 1621 gactgatgct tccaattcgc acaactctca aaccgaagtt gccgctacct tggcataccg 1680
 1681 cttcggcaac gtaacgcccc gagtttctta cgcccacggc ttcaaaggtt tggttgatga 1740
 1741 tgcagacata ggcaacgaat acgaccaagt ggttgtcggt gcggaatacg acttctccaa 1800
 1801 acgcacttct gccttggttt ctgccggttg gttgcaagaa ggcaaaggcg aaaacaaatt 1860
 1861 cgtagcgact gccggcggtg ttggtctgcg ccacaaattc taaGAATTCC CTTAGACATG 1920
 1921 ACTGTTCCTC AGTTCAAGTT GGGCATTACG AGAAGACCGG TCTTGCTAGA TTCTAATCAA 1980
 1981 GAGGATGTCA GAATGCCATT TGCCTGAGAG ATGCAGGCTT CATTTTTGAT ACTTTTTTAT 2040
 2041 TTGTAACCTA TATAGTATAG GATTTTTTTT GTCATTTTGT TTCTTCTCGT ACGAGCTTGC 2100
 2101 TCCTGATCAG CCTATCTCGC AGCTGATGAA TATCTTGTGG TAGGGGTTTG GGAAAATCAT 2160
 2161 TCGAGTTTGA TGTTTTTCTT GGTATTTCCC ACTCCTCTTC AGAGTACAGA AGATTAAGTG 2220
 2221 AGAAGTTCGT TTGTGCAAGC TTATCGATAA GCTTTAATGC GGTAGTTTAT CACAGTTAAA 2280
 2281 TTGCTAACGC AGTCAGGCAC CGTGTATGAA ATCTAACAAT GCGCTCATCG TCATCCTCGG 2340
 2341 CACCGTCACC CTGGATGCTG TAGGCATAGG CTTGGTTATG CCGGTACTGC CGGGCCTCTT 2400
 2401 GCGGGATATC GTCCATTCCG ACAGCATCGC CAGTCACTAT GGCGTGCTGC TAGCGCTATA 2460
 2461 TGCGTTGATG CAATTTCTAT GCGCACCCGT TCTCGGAGCA CTGTCCGACC GCTTTGGCCG 2520
 2521 CCGCCCAGTC CTGCTCGCTT CGCTACTTGG AGCCACTATC GACTACGCGA TCATGGCGAC 2580
```

FIG.16A

2581 CACACCCGTC CTGTGGATCT ATCGAATCTA AATGTAAGTT AAAATCTCTA AATAATTAAA 2640
2641 TAAGTCCCAG TTTCTCCATA CGAACCTTAA CAGCATTGCG GTGAGCATCT AGACCTTCAA 2700
2701 CAGCAGCCAG ATCCATCACT GCTTGGCCAA TATGTTTCAG TCCCTCAGGA GTTACGTCTT 2760
2761 GTGAAGTGAT GAACTTCTGG AAGGTTGCAG TGTTAACTCC GCTGTATTGA CGGGCATATC 2820
2821 CGTACGTTGG CAAAGTGTGG TTGGTACCGG AGGAGTAATC TCCACAACTC TCTGGAGAGT 2880
2881 AGGCACCAAC AAACACAGAT CCAGCGTGTT GTACTTGATC AACATAAGAA GAAGCATTCT 2940
2941 CGATTTGCAG GATCAAGTGT TCAGGAGCGT ACTGATTGGA CATTTCCAAA GCCTGCTCGT 3000
3001 AGGTTGCAAC CGATAGGGTT GTAGAGTGTG CAATACACTT GCGTACAATT TCAACCCTTG 3060
3061 GCAACTGCAC AGCTTGGTTG TGAACAGCAT CTTCAATTCT GGCAAGCTCC TTGTCTGTCA 3120
3121 TATCGACAGC CAACAGAATC ACCTGGGAAT CAATACCATG TTCAGCTTGA GACAGAAGGT 3180
3181 CTGAGGCAAC GAAATCTGGA TCAGCGTATT TATCAGCAAT AACTAGAACT TCAGAAGGCC 3240
3241 CAGCAGGCAT GTCAATACTA CACAGGGCTG ATGTGTCATT TTGAACCATC ATCTTGGCAG 3300
3301 CAGTAACGAA CTGGTTTCCT GGACCAAATA TTTTGTCACA CTTAGGAACA GTTTCTGTTC 3360
3361 CGTAAGCCAT AGCAGCTACT GCCTGGGCGC CTCCTGCTAG CACGATACAC TTAGCACCAA 3420
3421 CCTTGTGGGC AACGTAGATG ACTTCTGGGG TAAGGGTACC ATCCTTCTTA GGTGGAGATG 3480
3481 CAAAAACAAT TTCTTTGCAA CCAGCAACTT TGGCAGGAAC ACCCAGCATC AGGGAAGTGG 3540
3541 AAGGCAGAAT TGCGGTTCCA CCAGGAATAT AGAGGCCAAC TTTCTCAATA GGTCTTGCAA 3600
3601 AACGAGAGCA GACTACACCA GGGCAAGTCT CAACTTGCAA CGTCTCCGTT AGTTGAGCTT 3660
3661 CATGGAATTT CCTGACGTTA TCTATAGAGA GATCAATGGC TCTCTTAACG TTATCTGGCA 3720
3721 ATTGCATAAG TTCCTCTGGG AAAGGAGCTT CTAACACAGG TGTCTTCAAA GCGACTCCAT 3780
3781 CAAACTTGGC AGTTAGTTCT AAAAGGGCTT TGTCACCATT TTGACGAACA TTGTCGACAA 3840

FIG. 16A-1

3841 TTGGTTTGAC TAATTCCATA ATCTGTTCCG TTTTCTGGAT AGGACGACGA AGGGCATCTT 3900
3901 CAATTTCTTG TGAGGAGGCC TTAGAAACGT CAATTTTGCA CAATTCAATA CGACCTTCAG 3960
3961 AAGGGACTTC TTTAGGTTTG GATTCTTCTT TAGGTTGTTC CTTGGTGTAT CCTGGCTTGG 4020
4021 CATCTCCTTT CCTTCTAGTG ACCTTTAGGG ACTTCATATC CAGGTTTCTC TCCACCTCGT 4080
4081 CCAACGTCAC ACCGTACTTG GCACATCTAA CTAATGCAAA ATAAAATAAG TCAGCACATT 4140
4141 CCCAGGCTAT ATCTTCCTTG GATTTAGCTT CTGCAAGTTC ATCAGCTTCC TCCCTAATTT 4200
4201 TAGCGTTCAA CAAAACTTCG TCGTCAAATA ACCGTTTGGT ATAAGAACCT TCTGGAGCAT 4260
4261 TGCTCTTACG ATCCCACAAG GTGGCTTCCA TGGCTCTAAG ACCCTTTGAT TGGCCAAAAC 4320
4321 AGGAAGTGCG TTCCAAGTGA CAGAAACCAA CACCTGTTTG TTCAACCACA AATTTCAAGC 4380
4381 AGTCTCCATC ACAATCCAAT TCGATACCCA GCAACTTTTG AGTTGCTCCA GATGTAGCAC 4440
4441 CTTTATACCA CAAACCGTGA CGACGAGATT GGTAGACTCC AGTTTGTGTC CTTATAGCCT 4500
4501 CCGGAATAGA CTTTTTGGAC GAGTACACCA GGCCCAACGA GTAATTAGAA GAGTCAGCCA 4560
4561 CCAAAGTAGT GAATAGACCA TCGGGGCGGT CAGTAGTCAA AGACGCCAAC AAAATTTCAC 4620
4621 TGACAGGGAA CTTTTTGACA TCTTCAGAAA GTTCGTATTC AGTAGTCAAT TGCCGAGCAT 4680
4681 CAATAATGGG GATTATACCA GAAGCAACAG TGGAAGTCAC ATCTACCAAC TTTGCGGTCT 4740
4741 CAGAAAAAGC ATAAACAGTT CTACTACCGC CATTAGTGAA ACTTTTCAAA TCGCCCAGTG 4800
4801 GAGAAGAAAA AGGCACAGCG ATACTAGCAT TAGCGGGCAA GGATGCAACT TTATCAACCA 4860
4861 GGGTCCTATA GATAACCCTA GCGCCTGGGA TCATCCTTTG GACAACTCTT TCTGCCAAAT 4920
4921 CTAGGTCCAA AATCACTTCA TTGATACCAT TATTGTACAA CTTGAGCAAG TTGTCGATCA 4980
4981 GCTCCTCAAA TTGGTCCTCT GTAACGGATG ACTCAACTTG CACATTAACT TGAAGCTCAG 5040
5041 TCGATTGAGT GAACTTGATC AGGTTGTGCA GCTGGTCAGC AGCATAGGGA AACACGGCTT 5100
5101 TTCCTACCAA ACTCAAGGAA TTATCAAACT CTGCAACACT TGCGTATGCA GGTAGCAAGG 5160
5161 GAAATGTCAT ACTTGAAGTC GGACAGTGAG TGTAGTCTTG AGAAATTCTG AAGCCGTATT 5220
5221 TTTATTATCA GTGAGTCAGT CATCAGGAGA TCCTCTACGC CGGACGCATC GTGGCCGGCA 5280
5281 TCACCGGCGC CACAGGTGCG GTTGCTGGCG CCTATATCGC CGACATCACC GATGGGGAAG 5340
5341 ATCGGGCTCG CCACTTCGGG CTCATGAGCG CTTGTTTCGG CGTGGGTATG GTGGCAGGCC 5400
5401 CCGTGGCCGG GGGACTGTTG GGCGCCATCT CCTTGCATGC ACCATTCCTT GCGGCGGCGG 5460
5461 TGCTCAACGG CCTCAACCTA CTACTGGGCT GCTTCCTAAT GCAGGAGTCG CATAAGGGAG 5520
5521 AGCGTCGAGT ATCTATGATT GGAAGTATGG GAATGGTGAT ACCCGCATTC TTCAGTGTCT 5580
5581 TGAGGTCTCC TATCAGATTA TGCCCAACTA AAGCAACCGG AGGAGGAGAT TTCATGGTAA 5640
5641 ATTTCTCTGA CTTTTGGTCA TCAGTAGACT CGAACTGTGA GACTATCTCG GTTATGACAG 5700
5701 CAGAAATGTC CTTCTTGGAG ACAGTAAATG AAGTCCCACC AATAAAGAAA TCCTTGTTAT 5760
5761 CAGGAACAAA CTTCTTGTTT CGAACTTTTT CGGTGCCTTG AACTATAAAA TGTAGAGTGG 5820
5821 ATATGTCGGG TAGGAATGGA GCGGGCAAAT GCTTACCTTC TGGACCTTCA AGAGGTATGT 5880
5881 AGGGTTTGTA GATACTGATG CCAACTTCAG TGACAACGTT GCTATTTCGT TCAAACCATT 5940
5941 CCGAATCCAG AGAAATCAAA GTTGTTTGTC TACTATTGAT CCAAGCCAGT GCGGTCTTGA 6000
6001 AACTGACAAT AGTGTGCTCG TGTTTTGAGG TCATCTTTGT ATGAATAAAT CTAGTCTTTG 6060
6061 ATCTAAATAA TCTTGACGAG CCAAGGCGAT AAATACCCAA ATCTAAAACT CTTTTAAAAC 6120
6121 GTTAAAAGGA CAAGTATGTC TGCCTGTATT AAACCCCAAA TCAGCTCGTA GTCTGATCCT 6180
6181 CATCAACTTG AGGGGCACTA TCTTGTTTTA GAGAAATTTG CGGAGATGCG ATATCGAGAA 6240
6241 AAAGGTACGC TGATTTTAAA CGTGAAATTT ATCTCAAGAT CgcggccGCG ATCTCGAATA 6300
6301 ATAACTGTTA TTTTTCAGTG TTCCCGATCT GCGTCTATTT CACAATACCA ACATGAGTCA 6360
6361 GCTTATCGAT GATAAGCTGT CAAACATGAG AATTAATTCG ATGATAAGCT GTCAAACATG 6420

FIG.16B

6421 AGAAATCTTG AAGACGAAAG GGCCTCGTGA TACGCCTATT TTTATAGGTT AATGTCATGA 6840
6481 TAATAATGGT TTCTTAGACG TCAGGTGGCA CTTTTCGGGG AAATGTGCGC GGAACCCCTA 6540
6541 TTTGTTTATT TTTCTAAATA CATTCAAATA TGTATCCGCT CATGAGACAA TAACCCTGAT 6600
6601 AAATGCTTCA ATAATATTGA AAAAGGAAGA GTATGAGTAT TCAACATTTC CGTGTCGCCC 6660
6661 TTATTCCCTT TTTTGCGGCA TTTTGCCTTC CTGTTTTTGC TCACCCAGAA ACGCTGGTGA 6720
6721 AAGTAAAAGA TGCTGAAGAT CAGTTGGGTG CACGAGTGGG TTACATCGAA CTGGATCTCA 6780
6781 ACAGCGGTAA GATCCTTGAG AGTTTTCGCC CCGAAGAACG TTTTCCAATG ATGAGCACTT 6840
6841 TTAAAGTTCT GCTATGTGGC GCGGTATTAT CCCGTGTTGA CGCCGGGCAA GAGCAACTCG 6900
6901 GTCGCCGCAT ACACTATTCT CAGAATGACT TGGTTGAGTA CTCACCAGTC ACAGAAAAGC 6960
6961 ATCTTACGGA TGGCATGACA GTAAGAGAAT TATGCAGTGC TGCCATAACC ATGAGTGATA 7020
7021 ACACTGCGGC CAACTTACTT CTGACAACGA TCGGAGGACC GAAGGAGCTA ACCGCTTTTT 7080
7081 TGCACAACAT GGGGGATCAT GTAACTCGCC TTGATCGTTG GGAACCGGAG CTGAATGAAG 7140
7141 CCATACCAAA CGACGAGCGT GACACCACGA TGCCTGCAGC AATGGCAACA ACGTTGCGCA 7200
7201 AACTATTAAC TGGCGAACTA CTTACTCTAG CTTCCCGGCA ACAATTAATA GACTGGATGG 7260
7261 AGGCGGATAA AGTTGCAGGA CCACTTCTGC GCTCGGCCCT TCCGGCTGGC TGGTTTATTG 7320
7321 CTGATAAATC TGGAGCCGGT GAGCGTGGGT CTCGCGGTAT CATTGCAGCA CTGGGGCCAG 7380
7381 ATGGTAAGCC CTCCCGTATC GTAGTTATCT ACACGACGGG GAGTCAGGCA ACTATGGATG 7440
7441 AACGAAATAG ACAGATCGCT GAGATAGGTG CCTCACTGAT TAAGCATTGG TAACTGTCAG 7500
7501 ACCAAGTTTA CTCATATATA CTTTAGATTG ATTTAAATTG TAAACGTTAA TATTTTGTTA 7560
7561 AAATTCGCGT TAAATTTTTG TTAAATCAGC TCATTTTTTA ACCAATAGGC CGAAATCGGC 7620
7621 AAAATCCCTT ATAAATCAAA AGAATAGACC GAGATAGGGT TGAGTGTTGT TCCAGTTTGG 7680
7681 AACAAGAGTC CACTATTAAA GAACGTGGAC TCCAACGTCA AAGGGCGAAA AACCGTCTAT 7740
7741 CAGGGCGATG GCCCACTACG TGAACCATCA CCCTAATCAA GTTTTTTGGG GTCGAGGTGC 7800
7801 CGTAAAGCAC TAAATCGGAA CCCTAAAGGG AGCCCCCGAT TTAGAGCTTG ACGGGGAAAG 7860
7861 CCGGCGAACG TGGCGAGAAA GGAAGGGAAG AAAGCGAAAG GAGCGGGCGC TAGGGCGCTG 7920
7921 GCAAGTGTAG CGGTCACGCT GCGCGTAACC ACCACACCCG CCGCGCTTAA TGCGCCGCTA 7980
7981 CAGGGCGCGT AAAAGGATCT AGGTGAAGAT CCTTTTTGAT AATCTCATGA CCAAAATCCC 8040
8041 TTAACGTGAG TTTTCGTTCC ACTGAGCGTC AGACCCCGTA GAAAAGATCA AAGGATCTTC 8100
8101 TTGAGATCCT TTTTTTCTGC GCGTAATCTG CTGCTTGCAA ACAAAAAAAC CACCGCTACC 8160

FIG.16B-1

```
8161 AGCGGTGGTT TGTTTGCCGG ATCAAGAGCT ACCAACTCTT TTTCCGAAGG TAACTGGCTT 8220
8221 CAGCAGAGCG CAGATACCAA ATACTGTCCT TCTAGTGTAG CCGTAGTTAG GCCACCACTT 8280
8281 CAAGAACTCT GTAGCACCGC CTACATACCT CGCTCTGCTA ATCCTGTTAC CAGTGGCTGC 8340
8341 TGCCAGTGGC GATAAGTCGT GTCTTACCGG GTTGGACTCA AGACGATAGT TACCGGATAA 8400
8401 GGCGCAGCGG TCGGGCTGAA CGGGGGGTTC GTGCACACAG CCCAGCTTGG AGCGAACGAC 8460
8461 CTACACCGAA CTGAGATACC TACAGCGTGA GCATTGAGAA AGCGCCACGC TTCCCGAAGG 8520
8521 GAGAAAGGCG GACAGGTATC CGGTAAGCGG CAGGGTCGGA ACAGGAGAGC GCACGAGGGA 8580
8581 GCTTCCAGGG GGAAACGCCT GGTATCTTTA TAGTCCTGTC GGGTTTCGCC ACCTCTGACT 8640
8641 TGAGCGTCGA TTTTTGTGAT GCTCGTCAGG GGGGCGGAGC CTATGGAAAA ACGCCAGCAA 8700
8701 CGCGGCCTTT TTACGGTTCC TGGCCTTTTG CTGGCCTTTT GCTCACATGT TCTTTCCTGC 8760
8761 GTTATCCCCT GATTCTGTGG ATAACCGTAT TACCGCCTTT GAGTGAGCTG ATACCGCTCG 8820
8821 CCGCAGCCGA ACGACCGAGC GCAGCCAGTC AGTGAGCGAG GAAGCGGAAG AGCGCCTGAT 8880
8881 GCGGTATTTT CTCCTTACGC ATCTGTGCGG TATTTCACAC CGCATATGGT GCACTCTCAG 8940
8941 TACAATCTGC TCTGATGCCG CATAGTTAAG CCAGTATACA CTCCGCTATC GCTACGTGAC 9000
9001 TGGGTCATGG CTGCGCCCCG ACACCCGCCA ACACCCGCTG ACGCGCCCTG ACGGGCTTGT 9060
9061 CTGCTCCCGG CATCCGCTTA CAGACAAGCT GTGACCGTCT CCGGGAGCTG CATGTGTCAG 9120
9121 AGGTTTTCAC CGTCATCACC GAAACGCGCG AGGCAG                         9156
         |  10     |  20     |  30     |  40     |  50     |  60
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FIG.16C

```
      |    10      |    20      |    30      |    40      |    50      |    60
    1 AGATCTAACA TCCAAAGACG AAAGGTTGAA TGAAACCTTT TTGCCATCCG ACATCCACAG 60
   61 GTCCATTCTC ACACATAAGT GCCAAACGCA ACAGGAGGGG ATACACTAGC AGCAGACCGT 120
  121 TGCAAACGCA GGACCTCCAC TCCTCTTCTC CTCAACACCC ACTTTTGCCA TCGAAAAACC 180
  181 AGCCCAGTTA TTGGGCTTGA TTGGAGCTCG CTCATTCCAA TTCCTTCTAT TAGGCTACTA 240
  241 ACACCATGAC TTTATTAGCC TGTCTATCCT GGCCCCCCTG GCGAGGTCAT GTTTGTTTAT 300
  301 TTCCGAATGC AACAAGCTCC GCATTACACC CGAACATCAC TCCAGATGAG GGCTTTCTGA 360
  361 GTGTGGGGTC AAATAGTTTC ATGTTCCCAA ATGGCCCAAA ACTGACAGTT TAAACGCTGT 420
  421 CTTGGAACCT AATATGACAA AAGCGTGATC TCATCCAAGA TGAACTAAGT TTGGTTCGTT 480
  481 GAAATGCTAA CGGCCAGTTG GTCAAAAAGA AACTTCCAAA AGTCGCCATA CCGTTTGTCT 540
  541 TGTTTGGTAT TGATTGACGA ATGCTCAAAA ATAATCTCAT TAATGCTTAG CGCAGTCTCT 600
  601 CTATCGCTTC TGAACCCGGT GGCACCTGTG CCGAAACGCA AATGGGGAAA CAACCCGCTT 660
  661 TTTGGATGAT TATGCATTGT CCTCCACATT GTATGCTTCC AAGATTCTGG TGGGAATACT 720
  721 GCTGATAGCC TAACGTTCAT GATCAAAATT TAACTGTTCT AACCCCTACT TGACAGGCAA 780
  781 TATATAAACA GAAGGAAGCT GCCCTGTCTT AAACCTTTTT TTTTATCATC ATTATTAGCT 840
  841 TACTTTCATA ATTGCGACTG GTTCCAATTG ACAAGCTTTT GATTTTAACG ACTTTTAACG 900
  901 ACAACTTGAG AAGATCAAAA AACAACTAAT TATTCGAAAC GATGTTCTCT CCAATTTTGT 960
  961 CCTTGGAAAT TATTTTAGCT TTGGCTACTT TGCAATCTGT CTTCGCTCGA gacgtcactt 1020
 1021 tgtacggtac tattaaggct ggtgttgaga cttcccgctc tgtatttcac cagaacggcc 1080
 1081 aagttactga agttacaacc gctaccggca tcgttgattt gggttcgaaa atcggcttca 1140
 1141 aaggccaaga agacctcggt aacggcctga aagccatttg gcaggttgag caaaaagcat 1200
 1201 ctatcgccgg tactgactcc ggttggggca accgccaatc cttcatcggc ttgaaaggcg 1260
 1261 gcttcggtaa attgcgcgtc ggtcgtttga acagcgtcct gaaagacacc ggcgacatca 1320
 1321 atccttggga tagcaaaagc gactatttgg gtgtaaacaa aattgccgaa cccgaggcac 1380
 1381 gcctcatttc cgtacgctac gattctcccg aatttgccgg cctcagcggc agcgtacaat 1440
 1441 acgcgcttaa cgacaatgca ggcagacata acagcgaatc ttaccacgcc ggcttcaact 1500
 1501 acaaaaacgg tggcttcttc gtgcaatatg Gcggtgccta taaaagacat catcaagtgc 1560
 1561 aagagggctt gaatattgag aaataccaga ttcaccgttt ggtcagcggt tacgacaatg 1620
 1621 atgccctgta cgcttccgta gccgtacagc aacaagacgc gaaactgact gatgcttcca 1680
 1681 attcgcacaa ctctcaaacc gaagttgccg ctaccttggc ataccgcttc ggcaacgtaa 1740
 1741 cgccccgagt ttcttacgcc cacggcttca aaggtttggt tgatgatgca gacataggca 1800
 1801 acgaatacga ccaagtggtt gtcggtgcgg aatacgactt ctccaaacgc acttctgcct 1860
 1861 tggtttctgc cggttggttg caagaaggca aaggcgaaaa caaattcgta gcgactgccg 1920
 1921 gcggtgtCgg tctgcgCcac aaattctaaT CTGGATCCTT AGACATGACT GTTCCTCAGT 1980
 1981 TCAAGTTGGG CATTACGAGA AGACCGGTCT TGCTAGATTC TAATCAAGAG GATGTCAGAA 2040
 2041 TGCCATTTGC CTGAGAGATG CAGGCTTCAT TTTTGATACT TTTTTATTTG TAACCTATAT 2100
 2101 AGTATAGGAT TTTTTTTGTC ATTTTGTTTC TTCTCGTACG AGCTTGCTCC TGATCAGCCT 2160
 2161 ATCTCGCAGC TGATGAATAT CTTGTGGTAG GGGTTTGGGA AAATCATTCG AGTTTGATGT 2220
 2221 TTTTCTTGGT ATTTCCCACT CCTCTTCAGA GTACAGAAGA TTAAGTGAGA AGTTCGTTTG 2280
 2281 TGCAAGCTTA TCGATAAGCT TTAATGCGGT AGTTTATCAC AGTTAAATTG CTAACGCAGT 2340
 2341 CAGGCACCGT GTATGAAATC TAACAATGCG CTCATCGTCA TCCTCGGCAC CGTCACCCTG 2400
 2401 GATGCTGTAG GCATAGGCTT GGTTATGCCG GTACTGCCGG GCCTCTTGCG GGATATCGTC 2460
 2461 CATTCCGACA GCATCGCCAG TCACTATGGC GTGCTGCTAG CGCTATATGC GTTGATGCAA 2520
```

FIG. 17A

2521 TTTCTATGCG CACCCGTTCT CGGAGCACTG TCCGACCGCT TTGGCCGCCG CCCAGTCCTG 2580
2581 CTCGCTTCGC TACTTGGAGC CACTATCGAC TACGCGATCA TGGCGACCAC ACCCGTCCTG 2640
2641 TGGATCTATC GAATCTAAAT GTAAGTTAAA ATCTCTAAAT AATTAAATAA GTCCCAGTTT 2700
2701 CTCCATACGA ACCTTAACAG CATTGCGGTG AGCATCTAGA CCTTCAACAG CAGCCAGATC 2760
2761 CATCACTGCT TGGCCAATAT GTTTCAGTCC CTCAGGAGTT ACGTCTTGTG AAGTGATGAA 2820
2821 CTTCTGGAAG GTTGCAGTGT TAACTCCGCT GTATTGACGG GCATATCCGT ACGTTGGCAA 2880
2881 AGTGTGGTTG GTACCGGAGG AGTAATCTCC ACAACTCTCT GGAGAGTAGG CACCAACAAA 2940
2941 CACAGATCCA GCGTGTTGTA CTTGATCAAC ATAAGAAGAA GCATTCTCGA TTTGCAGGAT 3000
3001 CAAGTGTTCA GGAGCGTACT GATTGGACAT TTCCAAAGCC TGCTCGTAGG TTGCAACCGA 3060
3061 TAGGGTTGTA GAGTGTGCAA TACACTTGCG TACAATTTCA ACCCTTGGCA ACTGCACAGC 3120
3121 TTGGTTGTGA ACAGCATCTT CAATTCTGGC AAGCTCCTTG TCTGTCATAT CGACAGCCAA 3180
3181 CAGAATCACC TGGGAATCAA TACCATGTTC AGCTTGAGAC AGAAGGTCTG AGGCAACGAA 3240
3241 ATCTGGATCA GCGTATTTAT CAGCAATAAC TAGAACTTCA GAAGGCCCAG CAGGCATGTC 3300
3301 AATACTACAC AGGGCTGATG TGTCATTTTG AACCATCATC TTGGCAGCAG TAACGAACTG 3360
3361 GTTTCCTGGA CCAAATATTT TGTCACACTT AGGAACAGTT TCTGTTCCGT AAGCCATAGC 3420
3421 AGCTACTGCC TGGGCGCCTC CTGCTAGCAC GATACACTTA GCACCAACCT TGTGGGCAAC 3480
3481 GTAGATGACT TCTGGGGTAA GGGTACCATC CTTCTTAGGT GGAGATGCAA AAACAATTTC 3540
3541 TTTGCAACCA GCAACTTTGG CAGGAACACC CAGCATCAGG GAAGTGGAAG GCAGAATTGC 3600
3601 GGTTCCACCA GGAATATAGA GGCCAACTTT CTCAATAGGT CTTGCAAAAC GAGAGCAGAC 3660
3661 TACACCAGGG CAAGTCTCAA CTTGCAACGT CTCCGTTAGT TGAGCTTCAT GGAATTTCCT 3720
3721 GACGTTATCT ATAGAGAGAT CAATGGCTCT CTTAACGTTA TCTGGCAATT GCATAAGTTC 3780
3781 CTCTGGGAAA GGAGCTTCTA ACACAGGTGT CTTCAAAGCG ACTCCATCAA ACTTGGCAGT 3840

FIG.17A-1

3841 TAGTTCTAAA AGGGCTTTGT CACCATTTTG ACGAACATTG TCGACAATTG GTTTGACTAA 3900
3901 TTCCATAATC TGTTCCGTTT TCTGGATAGG ACGACGAAGG GCATCTTCAA TTTCTTGTGA 3960
3961 GGAGGCCTTA GAAACGTCAA TTTTGCACAA TTCAATACGA CCTTCAGAAG GGACTTCTTT 4020
4021 AGGTTTGGAT TCTTCTTTAG GTTGTTCCTT GGTGTATCCT GGCTTGGCAT CTCCTTTCCT 4080
4081 TCTAGTGACC TTTAGGGACT TCATATCCAG GTTTCTCTCC ACCTCGTCCA ACGTCACACC 4140
4141 GTACTTGGCA CATCTAACTA ATGCAAAATA AAATAAGTCA GCACATTCCC AGGCTATATC 4200
4201 TTCCTTGGAT TTAGCTTCTG CAAGTTCATC AGCTTCCTCC CTAATTTTAG CGTTCAACAA 4260
4261 AACTTCGTCG TCAAATAACC GTTTGGTATA AGAACCTTCT GGAGCATTGC TCTTACGATC 4320
4321 CCACAAGGTG GCTTCCATGG CTCTAAGACC CTTTGATTGG CCAAAACAGG AAGTGCGTTC 4380
4381 CAAGTGACAG AAACCAACAC CTGTTTGTTC AACCACAAAT TTCAAGCAGT CTCCATCACA 4440
4441 ATCCAATTCG ATACCCAGCA ACTTTTGAGT TGCTCCAGAT GTAGCACCTT TATACCACAA 4500
4501 ACCGTGACGA CGAGATTGGT AGACTCCAGT TTGTGTCCTT ATAGCCTCCG GAATAGACTT 4560
4561 TTTGGACGAG TACACCAGGC CCAACGAGTA ATTAGAAGAG TCAGCCACCA AAGTAGTGAA 4620
4621 TAGACCATCG GGGCGGTCAG TAGTCAAAGA CGCCAACAAA ATTTCACTGA CAGGGAACTT 4680
4681 TTTGACATCT TCAGAAAGTT CGTATTCAGT AGTCAATTGC CGAGCATCAA TAATGGGGAT 4740
4741 TATACCAGAA GCAACAGTGG AAGTCACATC TACCAACTTT GCGGTCTCAG AAAAAGCATA 4800
4801 AACAGTTCTA CTACCGCCAT TAGTGAAACT TTTCAAATCG CCCAGTGGAG AAGAAAAAGG 4860
4861 CACAGCGATA CTAGCATTAG CGGGCAAGGA TGCAACTTTA TCAACCAGGG TCCTATAGAT 4920
4921 AACCCTAGCG CCTGGGATCA TCCTTTGGAC AACTCTTTCT GCCAAATCTA GGTCCAAAAT 4980
4981 CACTTCATTG ATACCATTAT TGTACAACTT GAGCAAGTTG TCGATCAGCT CCTCAAATTG 5040
5041 GTCCTCTGTA ACGGATGACT CAACTTGCAC ATTAACTTGA AGCTCAGTCG ATTGAGTGAA 5100
5101 CTTGATCAGG TTGTGCAGCT GGTCAGCAGC ATAGGGAAAC ACGGCTTTTC CTACCAAACT 5160
5161 CAAGGAATTA TCAAACTCTG CAACACTTGC GTATGCAGGT AGCAAGGGAA ATGTCATACT 5220
5221 TGAAGTCGGA CAGTGAGTGT AGTCTTGAGA AATTCTGAAG CCGTATTTTT ATTATCAGTG 5280
5281 AGTCAGTCAT CAGGAGATCC TCTACGCCGG ACGCATCGTG GCCGGCATCA CCGGCGCCAC 5340
5341 AGGTGCGGTT GCTGGCGCCT ATATCGCCGA CATCACCGAT GGGGAAGATC GGGCTCGCCA 5400
5401 CTTCGGGCTC ATGAGCGCTT GTTTCGGCGT GGGTATGGTG GCAGGCCCCG TGGCCGGGGG 5460
5461 ACTGTTGGGC GCCATCTCCT TGCATGCACC ATTCCTTGCG GCGGCGGTGC TCAACGGCCT 5520
5521 CAACCTACTA CTGGGCTGCT TCCTAATGCA GGAGTCGCAT AAGGGAGAGC GTCGAGTATC 5580
5581 TATGATTGGA AGTATGGGAA TGGTGATACC CGCATTCTTC AGTGTCTTGA GGTCTCCTAT 5640
5641 CAGATTATGC CCAACTAAAG CAACCGGAGG AGGAGATTTC ATGGTAAATT TCTCTGACTT 5700
5701 TTGGTCATCA GTAGACTCGA ACTGTGAGAC TATCTCGGTT ATGACAGCAG AAATGTCCTT 5760
5761 CTTGGAGACA GTAAATGAAG TCCCACCAAT AAAGAAATCC TTGTTATCAG GAACAAACTT 5820
5821 CTTGTTTCGA ACTTTTTCGG TGCCTTGAAC TATAAAATGT AGAGTGGATA TGTCGGGTAG 5880
5881 GAATGGAGCG GGCAAATGCT TACCTTCTGG ACCTTCAAGA GGTATGTAGG GTTTGTAGAT 5940
5941 ACTGATGCCA ACTTCAGTGA CAACGTTGCT ATTTCGTTCA AACCATTCCG AATCCAGAGA 6000
6001 AATCAAAGTT GTTTGTCTAC TATTGATCCA AGCCAGTGCG GTCTTGAAAC TGACAATAGT 6060
6061 GTGCTCGTGT TTTGAGGTCA TCTTTGTATG AATAAATCTA GTCTTTGATC TAAATAATCT 6120
6121 TGACGAGCCA AGGCGATAAA TACCCAAATC TAAAACTCTT TTAAAACGTT AAAAGGACAA 6180
6181 GTATGTCTGC CTGTATTAAA CCCCAAATCA GCTCGTAGTC TGATCCTCAT CAACTTGAGG 6240
6241 GGCACTATCT TGTTTTAGAG AAATTTGCGG AGATGCGATA TCGAGAAAAA GGTACGCTGA 6300
6301 TTTTAAACGT GAAATTTATC TCAAGATCTC TGCCTCGCGC GTTTCGGTGA TGACGGTGAA 6360
6361 AACCTCTGAC ACATGCAGCT CCCGGAGACG GTCACAGCTT GTCTGTAAGC GGATGCCGGG 6420

FIG.17B

6421 AGCAGACAAG CCCGTCAGGG CGCGTCAGCG GGTGTTGGCG GGTGTCGGGG CGCAGCCATG 6480
6481 ACCCAGTCAC GTAGCGATAG CGGAGTGTAT ACTGGCTTAA CTATGCGGCA TCAGAGCAGA 6540
6541 TTGTACTGAG AGTGCACCAT ATGCGGTGTG AAATACCGCA CAGATGCGTA AGGAGAAAAT 6600
6601 ACCGCATCAG GCGCTCTTCC GCTTCCTCGC TCACTGACTC GCTGCGCTCG GTCGTTCGGC 6660
6661 TGCGGCGAGC GGTATCAGCT CACTCAAAGG CGGTAATACG GTTATCCACA GAATCAGGGG 6720
6721 ATAACGCAGG AAAGAACATG TGAGCAAAAG GCCAGCAAAA GGCCAGGAAC CGTAAAAAGG 6780
6781 CCGCGTTGCT GGCGTTTTTC CATAGGCTCC GCCCCCCTGA CGAGCATCAC AAAAATCGAC 6840
6841 GCTCAAGTCA GAGGTGGCGA AACCCGACAG GACTATAAAG ATACCAGGCG TTTCCCCCTG 6900
6901 GAAGCTCCCT CGTGCGCTCT CCTGTTCCGA CCCTGCCGCT TACCGGATAC CTGTCCGCCT 6960
6961 TTCTCCCTTC GGGAAGCGTG GCGCTTTCTC AATGCTCACG CTGTAGGTAT CTCAGTTCGG 7020
7021 TGTAGGTCGT TCGCTCCAAG CTGGGCTGTG TGCACGAACC CCCCGTTCAG CCCGACCGCT 7080
7081 GCGCCTTATC CGGTAACTAT CGTCTTGAGT CCAACCCGGT AAGACACGAC TTATCGCCAC 7140
7141 TGGCAGCAGC CACTGGTAAC AGGATTAGCA GAGCGAGGTA TGTAGGCGGT GCTACAGAGT 7200
7201 TCTTGAAGTG GTGGCCTAAC TACGGCTACA CTAGAAGGAC AGTATTTGGT ATCTGCGCTC 7260
7261 TGCTGAAGCC AGTTACCTTC GGAAAAAGAG TTGGTAGCTC TTGATCCGGC AAACAAACCA 7320
7321 CCGCTGGTAG CGGTGGTTTT TTTGTTTGCA AGCAGCAGAT TACGCGCAGA AAAAAAGGAT 7380
7381 CTCAAGAAGA TCCTTTGATC TTTTCTACGG GGTCTGACGC TCAGTGGAAC GAAAACTCAC 7440
7441 GTTAAGGGAT TTTGGTCATG AGATTATCAA AAAGGATCTT CACCTAGATC CTTTTACGCG 7500
7501 CCCTGTAGCG GCGCATTAAG CGCGGCGGGT GTGGTGGTTA CGCGCAGCGT GACCGCTACA 7560
7561 CTTGCCAGCG CCCTAGCGCC CGCTCCTTTC GCTTTCTTCC CTTCCTTTCT CGCCACGTTC 7620
7621 GCCGGCTTTC CCCGTCAAGC TCTAAATCGG GGGCTCCCTT TAGGGTTCCG ATTTAGTGCT 7680
7681 TTACGGCACC TCGACCCCAA AAAACTTGAT TAGGGTGATG GTTCACGTAG TGGGCCATCG 7740
7741 CCCTGATAGA CGGTTTTTCG CCCTTTGACG TTGGAGTCCA CGTTCTTTAA TAGTGGACTC 7800
7801 TTGTTCCAAA CTGGAACAAC ACTCAACCCT ATCTCGGTCT ATTCTTTTGA TTTATAAGGG 7860
7861 ATTTTGCCGA TTTCGGCCTA TTGGTTAAAA AATGAGCTGA TTTAACAAAA ATTTAACGCG 7920
7921 AATTTTAACA AAATATTAAC GTTTACAATT TAAATCAATC TAAAGTATAT ATGAGTAAAC 7980
7981 TTGGTCTGAC AGTTACCAAT GCTTAATCAG TGAGGCACCT ATCTCAGCGA TCTGTCTATT 8040
8041 TCGTTCATCC ATAGTTGCCT GACTCCCCGT CGTGTAGATA ACTACGATAC GGGAGGGCTT 8100
8101 ACCATCTGGC CCCAGTGCTG CAATGATACC GCGAGACCCA CGCTCACCGG CTCCAGATTT 8160

FIG.17B-1

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8161 ATCAGCAATA AACCAGCCAG CCGGAAGGGC CGAGCGCAGA AGTGGTCCTG CAACTTTATC 8220
8221 CGCCTCCATC CAGTCTATTA ATTGTTGCCG GGAAGCTAGA GTAAGTAGTT CGCCAGTTAA 8280
8281 TAGTTTGCGC AACGTTGTTG CCATTGCTGC AGGCATCGTG GTGTCACGCT CGTCGTTTGG 8340
8341 TATGGCTTCA TTCAGCTCCG GTTCCCAACG ATCAAGGCGA GTTACATGAT CCCCCATGTT 8400
8401 GTGCAAAAAA GCGGTTAGCT CCTTCGGTCC TCCGATCGTT GTCAGAAGTA AGTTGGCCGC 8460
8461 AGTGTTATCA CTCATGGTTA TGGCAGCACT GCATAATTCT CTTACTGTCA TGCCATCCGT 8520
8521 AAGATGCTTT TCTGTGACTG GTGAGTACTC AACCAAGTCA TTCTGAGAAT AGTGTATGCG 8580
8581 GCGACCGAGT TGCTCTTGCC CGGCGTCAAC ACGGGATAAT ACCGCGCCAC ATAGCAGAAC 8640
8641 TTTAAAAGTG CTCATCATTG GAAAACGTTC TTCGGGGCGA AAACTCTCAA GGATCTTACC 8700
8701 GCTGTTGAGA TCCAGTTCGA TGTAACCCAC TCGTGCACCC AACTGATCTT CAGCATCTTT 8760
8761 TACTTTCACC AGCGTTTCTG GGTGAGCAAA AACAGGAAGG CAAAATGCCG CAAAAAAGGG 8820
8821 AATAAGGGCG ACACGGAAAT GTTGAATACT CATACTCTTC CTTTTTCAAT ATTATTGAAG 8880
8881 CATTTATCAG GGTTATTGTC TCATGAGCGG ATACATATTT GAATGTATTT AGAAAAATAA 8940
8941 ACAAATAGGG GTTCCGCGCA CATTTCCCCG AAAAGTGCCA CCTGACGTCT AAGAAACCAT 9000
9001 TATTATCATG ACATTAACCT ATAAAAATAG GCGTATCACG AGGCCCTTTC GTCTTCAAGA 9060
9061 TTTCTCATGT TTGACAGCTT ATCATCGAAT TAATTCTCAT GTTTGACAGC TTATCATCGA 9120
9121 TAAGCTGACT CATGTTGGTA TTGTGAAATA GACGCAGATC GGGAACACTG AAAAATAACA 9180
9181 GTTATTATTC G                                                      9191
        |  10     |  20     |  30     |  40     |  50     |  60
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FIG.17C

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        |   10      |   20      |   30      |   40      |   50      |   60
   1 AGATCTAACA TCCAAAGACG AAAGGTTGAA TGAAACCTTT TTGCCATCCG ACATCCACAG  60
  61 GTCCATTCTC ACACATAAGT GCCAAACGCA ACAGGAGGGG ATACACTAGC AGCAGACCGT 120
 121 TGCAAACGCA GGACCTCCAC TCCTCTTCTC CTCAACACCC ACTTTTGCCA TCGAAAAACC 180
 181 AGCCCAGTTA TTGGGCTTGA TTGGAGCTCG CTCATTCCAA TTCCTTCTAT TAGGCTACTA 240
 241 ACACCATGAC TTTATTAGCC TGTCTATCCT GGCCCCCCTG GCGAGGtTCA TGTTTGTTTA 300
 301 TTTCCGAATG CAACAAGCTC CGCATTACAC CCGAACATCA CTCCAGATGA GGGCTTTCTG 360
 361 AGTGTGGGGT CAAATAGTTT CATGTTCCCc AAATGGCCCA AAACTGACAG TTTAAACGCT 420
 421 GTCTTGGAAC CTAATATGAC AAAAGCGTGA TCTCATCCAA GATGAACTAA GTTTGGTTCG 480
 481 TTGAAATGCT AACGGCCAGT TGGTCAAAAA GAAACTTCCA AAAGTCGCCA TACCGTTTGT 540
 541 CTTGTTTGGT ATTGATTGAC GAATGCTCAA AAATAATCTC ATTAATGCTT AGCGCAGTCT 600
 601 CTCTATCGCT TCTGAACCCG GTGGCACCTG TGCCGAAACG CAAATGGGGA AACaCCCGCT 660
 661 TTTTGGATGA TTATGCATTG TcTCCACATT GTATGCTTCC AAGATTCTGG TGGGAATACT 720
 721 GCTGATAGCC TAACGTTCAT GATCAAAATT TAACTGTTCT AACCCCTACT TGACAGcAAT 780
 781 ATATAAACAG AAGGAAGCTG CCCTGTCTTA AACCTTTTTT TTTATCATCA TTATTAGCTT 840
 841 ACTTTCATAA TTGCGACTGG TTCCAATTGA CAAGCTTTTG ATTTTAACGA CTTTTAACGA 900
 901 CAACTTGAGA AGATCAAAAA ACAACTAATT ATTCGAAGGA TCCAAACGat gAGATTTCCT 960
 961 TCAATTTTTA CTGCAGTTTT ATTCGCAGCA TCCTCCGCAT TAGCTGCTCC AGTCAACACT 1020
1021 ACAACAGAAG ATGAAACGGC ACAAATTCCG GCTGAAGCTG TCATCGGTTA CTCAGATTTA 1080
1081 GAAGGGGATT TCGATGTTGC TGTTTTGCCA TTTTCCAACA GCACAAATAA CGGGTTATTG 1140
1141 TTTATAAATA CTACTATTGC CAGCATTGCT GCTAAAGAAG AAGGGGTATC TCTCGAGAAA 1200
1201 AGAGAGGCTG AAGCTTACGT AGAATTCgac gtcactttgt acggtactat taaggctggt 1260
1261 gttgagactt cccgctctgt atttcaccag aacggccaag ttactgaagt tacaaccgct 1320
1321 accggcatcg ttgatttggg ttcgaaaatc ggcttcaaag gccaagaaga cctcggtaac 1380
1381 ggcctgaaag ccatttggca ggttgagcaa aaagcatcta tcgccggtac tgactccggt 1440
1441 tggggcaacc gccaatcctt catcggcttg aaaggcggct tcggtaaatt gcgcgtcggt 1500
1501 cgtttgaaca gcgtcctgaa agacaccggc gacatcaatc cttgggatag caaaagcgac 1560
1561 tatttgggtg taaacaaaat tgccgaaccc gaggcacgcc tcatttccgt acgctacgat 1620
1621 tctcccgaat ttgccggcct cagcggcagc gtacaatacg cgcttaacga caatgcaggc 1680
1681 agacataaca gcgaatctta ccacgccggc ttcaactaca aaaacggtgg cttcttcgtg 1740
1741 caatatggcg gtgcctataa aagacatcat caagtgcaag agggcttgaa tattgagaaa 1800
1801 taccagattc accgtttggt cagcggttac gacaatgatg ccctgtacgc ttccgtagcc 1860
1861 gtacagcaac aagacgcgaa actgactgat gcttccaatt cgcacaactc tcaaaccgaa 1920
1921 gttgccgcta ccttggcata ccgcttcggc aacgtaacgc cccgagtttc ttacgcccac 1980
1981 ggcttcaaag gtttggttga tgatgcagac ataggcaacg aatacgacca agtggttgtc 2040
2041 ggtgcggaat acgacttctc caaacgcact tctgccttgg tttctgccgg ttggttgcaa 2100
2101 gaaggcaaag gcgaaaacaa attcgtagcg actgccggcg gtgttggtct gcgtcacaaa 2160
2161 ttctaaCCTA GGGCGGCCGC GAATTAAtTC GCCTTAGACA TGACTGTTCC TCAGTTCAAG 2220
2221 TTGGGCAcTT ACGAGAAGAC CGGTCTTGCT AGATTCTAAT CAAGAGGATG TCAGAATGCC 2280
2281 ATTTGCCTGA GAGATGCAGG CTTCATTTTT GATACTTTTT TATTTGTAAC CTATATAGTA 2340
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FIG.18A

2341 TAGGATTTTT TTTGTCATTT TGTTTCTTCT CGTACGAGCT TGCTCCTGAT CAGCCTATCT 2400
2401 CGCAGCTGAT GAATATCTTG TGGTAGGGGT TTGGGAAAAT CATTCGAGTT TGATGTTTTT 2460
2461 CTTGGTATTT CCCACTCCTC TTCAGAGTAC AGAAGATTAA GTGAGAAGTT CGTTTGTGCA 2520
2521 AGCTTATCGA TAAGCTTTAA TGCGGTAGTT TATCACAGTT AAATTGCTAA CGCAGTCAGG 2580
2581 CACCGTGTAT GAAATCTAAC AATGCGCTCA TCGTCATCCT CGGCACCGTC ACCCTGGATG 2640
2641 CTGTAGGCAT AGGCTTGGTT ATGCCGGTAC TGCCGGGCCT CTTGCGGGAT ATCGTCCATT 2700
2701 CCGACAGCAT CGCCAGTCAC TATGGCGTGC TGCTAGCGCT ATATGCGTTG ATGCAATTTC 2760
2761 TATGCGCACC CGTTCTCGGA GCACTGTCCG ACCGCTTTGG CCGCCGCCCA GTCCTGCTCG 2820
2821 CTTCGCTACT TGGAGCCACT ATCGACTACG CGATCATGGC GACCACACCC GTCCTGTGGA 2880
2881 TCTATCGAAT CTAAATGTAA GTTAAAATCT CTAAATAATT AAATAAGTCC CAGTTTCTCC 2940
2941 ATACGAACCT TAACAGCATT GCGGTGAGCA TCTAGACCTT CAACAGCAGC CAGATCCATC 3000
3001 ACTGCTTGGC CAATATGTTT CAGTCCCTCA GGAGTTACGT CTTGTGAAGT GATGAACTTC 3060
3061 TGGAAGGTTG CAGTGTTAAC TCCGCTGTAT TGACGGGCAT ATCCGTACGT TGGCAAAGTG 3120
3121 TGGTTGGTAC CGGAGGAGTA ATCTCCACAA CTCTCTGGAG AGTAGGCACC AACAAACACA 3180
3181 GATCCAGCGT GTTGTACTTG ATCAACATAA GAAGAAGCAT TCTCGATTTG CAGGATCAAG 3240
3241 TGTTCAGGAG CGTACTGATT GGACATTTCC AAAGCCTGCT CGTAGGTTGC AACCGATAGG 3300
3301 GTTGTAGAGT GTGCAATACA CTTGCGTACA ATTTCAACCC TTGGCAACTG CACAGCTTGG 3360
3361 TTGTGAACAG CATCTTCAAT TCTGGCAAGC TCCTTGTCTG TCATATCGAC AGCCAACAGA 3420
3421 ATCACCTGGG AATCAATACC ATGTTCAGCT TGAGACAGAA GGTCTGAGGC AACGAAATCT 3480
3481 GGATCAGCGT ATTTATCAGC AATAACTAGA ACTTCAGAAG GCCCAGCAGG CATGTCAATA 3540
3541 CTACACAGGG CTGATGTGTC ATTTTGAACC ATCATCTTGG CAGCAGTAAC GAACTGGTTT 3600
3601 CCTGGACCAA ATATTTTGTC ACACTTAGGA ACAGTTTCTG TTCCGTAAGC CATAGCAGCT 3660
3661 ACTGCCTGGG CGCCTCCTGC TAGCACGATA CACTTAGCAC CAACCTTGTG GGCAACGTAG 3720
3721 ATGACTTCTG GGGTAAGGGT ACCATCCTTC TTAGGTGGAG ATGCAAAAAC AATTTCTTTG 3780
3781 CAACCAGCAA CTTTGGCAGG AACACCCAGC ATCAGGGAAG TGGAAGGCAG AATTGCGGTT 3840

FIG.18A-1

3841 CCACCAGGAA TATAGAGGCC AACTTTCTCA ATAGGTCTTG CAAAACGAGA GCAGACTACA 3900
3901 CCAGGGCAAG TCTCAACTTG CAACGTCTCC GTTAGTTGAG CTTCATGGAA TTTCCTGACG 3960
3961 TTATCTATAG AGAGATCAAT GGCTCTCTTA ACGTTATCTG GCAATTGCAT AAGTTCCTCT 4020
4021 GGGAAAGGAG CTTCTAACAC AGGTGTCTTC AAAGCGACTC CATCAAACTT GGCAGTTAGT 4080
4081 TCTAAAAGGG CTTTGTCACC ATTTTGACGA ACATTGTCGA CAATTGGTTT GACTAATTCC 4140
4141 ATAATCTGTT CCGTTTTCTG GATAGGACGA CGAAGGGCAT CTTCAATTTC TTGTGAGGAG 4200
4201 GCCTTAGAAA CGTCAATTTT GCACAATTCA ATACGACCTT CAGAAGGGAC TTCTTTAGGT 4260
4261 TTGGATTCTT CTTTAGGTTG TTCCTTGGTG TATCCTGGCT TGGCATCTCC TTTCCTTCTA 4320
4321 GTGACCTTTA GGGACTTCAT ATCCAGGTTT CTCTCCACCT CGTCCAACGT CACACCGTAC 4380
4381 TTGGCACATC TAACTAATGC AAAATAAAAT AAGTCAGCAC ATTCCCAGGC TATATCTTCC 4440
4441 TTGGATTTAG CTTCTGCAAG TTCATCAGCT TCCTCCCTAA TTTTAGCGTT CAACAAAACT 4500
4501 TCGTCGTCAA ATAACCGTTT GGTATAAGAA CCTTCTGGAG CATTGCTCTT ACGATCCCAC 4560
4561 AAGGTGGCTT CCATGGCTCT AAGACCCTTT GATTGGCCAA AACAGGAAGT GCGTTCCAAG 4620
4621 TGACAGAAAC CAACACCTGT TTGTTCAACC ACAAATTTCA AGCAGTCTCC ATCACAATCC 4680
4681 AATTCGATAC CCAGCAACTT TTGAGTTGCT CCAGATGTAG CACCTTTATA CCACAAACCG 4740
4741 TGACGACGAG ATTGGTAGAC TCCAGTTTGT GTCCTTATAG CCTCCGGAAT AGACTTTTTG 4800
4801 GACGAGTACA CCAGGCCCAA CGAGTAATTA GAAGAGTCAG CCACCAAAGT AGTGAATAGA 4860
4861 CCATCGGGGC GGTCAGTAGT CAAAGACGCC AACAAAATTT CACTGACAGG GAACTTTTTG 4920
4921 ACATCTTCAG AAAGTTCGTA TTCAGTAGTC AATTGCCGAG CATCAATAAT GGGGATTATA 4980
4981 CCAGAAGCAA CAGTGGAAGT CACATCTACC AACTTTGCGG TCTCAGAAAA AGCATAAACA 5040
5041 GTTCTACTAC CGCCATTAGT GAAACTTTTC AAATCGCCCA GTGGAGAAGA AAAAGGCACA 5100
5101 GCGATACTAG CATTAGCGGG CAAGGATGCA ACTTTATCAA CCAGGGTCCT ATAGATAACC 5160
5161 CTAGCGCCTG GGATCATCCT TTGGACAACT CTTTCTGCCA AATCTAGGTC CAAAATCACT 5220
5221 TCATTGATAC CATTATTGTA CAACTTGAGC AAGTTGTCGA TCAGCTCCTC AAATTGGTCC 5280
5281 TCTGTAACGG ATGACTCAAC TTGCACATTA ACTTGAAGCT CAGTCGATTG AGTGAACTTG 5340
5341 ATCAGGTTGT GCAGCTGGTC AGCAGCATAG GGAAACACGG CTTTTCCTAC CAAACTCAAG 5400
5401 GAATTATCAA ACTCTGCAAC ACTTGCGTAT GCAGGTAGCA AGGGAAATGT CATACTTGAA 5460
5461 GTCGGACAGT GAGTGTAGTC TTGAGAAATT CTGAAGCCGT ATTTTTATTA TCAGTGAGTC 5520
5521 AGTCATCAGG AGATCCTCTA CGCCGGACGC ATCGTGGCCg acctgcaggt cGGCATCACC 5580
5581 GGCGCCACAG GTGCGGTTGC TGGCGCCTAT ATCGCCGACA TCACCGATGG GGAAGATCGG 5640
5641 GCTCGCCACT TCGGGCTCAT GAGCGCTTGT TTCGGCGTGG GTATGGTGGC AGGCCCCGTG 5700
5701 GCCGGGGGAC TGTTGGGCGC CATCTCCTTG CATGCACCAT TCCTTGCGGC GGCGGTGCTC 5760
5761 AACGGCCTCA ACCTACTACT GGGCTGCTTC CTAATGCAGG AGTCGCATAA GGGAGAGCGT 5820
5821 CGAGTATCTA TGATTGGAAG TATGGGAATG GTGATACCCG CATTCTTCAG TGTCTTGAGG 5880
5881 TCTCCTATCA GATTATGCCC AACTAAAGCA ACCGGAGGAG GAGATTTCAT GGTAAATTTC 5940
5941 TCTGACTTTT GGTCATCAGT AGACTCGAAC TGTGAGACTA TCTCGGTTAT GACAGCAGAA 6000
6001 ATGTCCTTCT TGGAGACAGT AAATGAAGTC CCACCAATAA AGAAATCCTT GTTATCAGGA 6060
6061 ACAAACTTCT TGTTTCGAAC TTTTTCGGTG CCTTGAACTA TAAAATGTAG AGTGGATATG 6120
6121 TCGGGTAGGA ATGGAGCGGG CAAATGCTTA CCTTCTGGAC CTTCAAGAGG TATGTAGGGT 6180

FIG.18B

6181 TTGTAGATAC TGATGCCAAC TTCAGTGACA ACGTTGCTAT TTCGTTCAAA CCATTCCGAA 6240
6241 TCCAGAGAAA TCAAAGTTGT TTGTCTACTA TTGATCCAAG CCAGTGCGGT CTTGAAACTG 6300
6301 ACAATAGTGT GCTCGTGTTT TGAGGTCATC TTTGTATGAA TAAATCTAGT CTTTGATCTA 6360
6361 AATAATCTTG ACGAGCCAAG GCGATAAATA CCCAAATCTA AAACTCTTTT AAAACGTTAA 6420
6421 AAGGACAAGT ATGTCTGCCT GTATTAAACC CCAAATCAGC TCGTAGTCTG ATCCTCATCA 6480
6481 ACTTGAGGGG CACTATCTTG TTTTAGAGAA ATTTGCGGAG ATGCGATATC GAGAAAAAGG 6540
6541 TACGCTGATT TTAAACGTGA AATTTATCTC AAGATCTCTG CCTCGCGCGT TTCGGTGATG 6600
6601 ACGGTGAAAA CCTCTGACAC ATGCAGCTCC CGGAGACGGT CACAGCTTGT CTGTAAGCGG 6660
6661 ATGCCGGGAG CAGACAAGCC CGTCAGGGCG CGTCAGCGGG TGTTGGCGGG TGTCGGGGCG 6720
6721 CAGCCATGAC CCAGTCACGT AGCGATAGCG GAGTGTATAC TGGCTTAACT ATGCGGCATC 6780
6781 AGAGCAGATT GTACTGAGAG TGCACCATAT GCGGTGTGAA ATACCGCACA GATGCGTAAG 6840
6841 GAGAAAATAC CGCATCAGGC GCTCTTCCGC TTCCTCGCTC ACTGACTCGC TGCGCTCGGT 6900
6901 CGTTCGGCTG CGGCGAGCGG TATCAGCTCA CTCAAAGGCG GTAATACGGT TATCCACAGA 6960
6961 ATCAGGGGAT AACGCAGGAA AGAACATGTG AGCAAAAGGC CAGCAAAAGG CCAGGAACCG 7020
7021 TAAAAAGGCC GCGTTGCTGG CGTTTTTCCA TAGGCTCCGC CCCCCTGACG AGCATCACAA 7080
7081 AAATCGACGC TCAAGTCAGA GGTGGCGAAA CCCGACAGGA CTATAAAGAT ACCAGGCGTT 7140
7141 TCCCCCTGGA AGCTCCCTCG TGCGCTCTCC TGTTCCGACC CTGCCGCTTA CCGGATACCT 7200
7201 GTCCGCCTTT CTCCCTTCGG GAAGCGTGGC GCTTTCTCAA TGCTCACGCT GTAGGTATCT 7260
7261 CAGTTCGGTG TAGGTCGTTC GCTCCAAGCT GGGCTGTGTG CACGAACCCC CCGTTCAGCC .7320
7321 CGACCGCTGC GCCTTATCCG GTAACTATCG TCTTGAGTCC AACCCGGTAA GACACGACTT 7380
7381 ATCGCCACTG GCAGCAGCCA CTGGTAACAG GATTAGCAGA GCGAGGTATG TAGGCGGTGC 7440
7441 TACAGAGTTC TTGAAGTGGT GGCCTAACTA CGGCTACACT AGAAGGACAG TATTTGGTAT 7500
7501 CTGCGCTCTG CTGAAGCCAG TTACCTTCGG AAAAAGAGTT GGTAGCTCTT GATCCGGCAA 7560
7561 ACAAACCACC GCTGGTAGCG GTGGTTTTTT TGTTTGCAAG CAGCAGATTA CGCGCAGAAA 7620
7621 AAAAGGATCT CAAGAAGATC CTTTGATCTT TTCTACGGGG TCTGACGCTC AGTGGAACGA 7680
7681 AAACTCACGT TAAGGGATTT TGGTCATGAG ATTATCAAAA AGGATCTTCA CCTAGATCCT 7740
7741 TTTaaAttaa aaatgaagtt ttaaatcaat ctaaagtata tatgagtaaa cttggtctga 7800
7801 cagttaccaa tgcttaatca gtgaggcacc tatctcagcg atctgtctat ttcgttcatc 7860
7861 catagttgcc tgactccccg tcgtgtagat aactacgata cgggagggct taccatctgg 7920
7921 ccccagtgct gcaatgatac cgcgagaccc acgctcaccg gctccagatt tatcagcaat 7980
7981 aaaccagcca gccggaaggg ccgagcgcag aagtggtcct gcaactttat ccgcctccat 8040
8041 ccagtctatt aattgttgcc gggaagctag agtaagtagt tcgccagtta atagtttgcg 8100
8101 caacgttgtt gccattgctg caggcatcgt ggtgtcacgc tcgtcgtttg gtatggcttc 8160

FIG.18B-1

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8161 attcagctcc ggttcccaac gatcaaggcg agttacatga tcccccatgt tgtgcaaaaa 8220
8221 agcggttagc tccttcggtc ctccgatcgt tgtcagaagt aagttggccg cagtgttatc 8280
8281 actcatggtt atggcagcac tgcataattc tcttactgtc atgccatccg taagatgctt 8340
8341 ttctgtgact ggtgagtact caaccaagtc attctgagaa tagtgtatgc ggcgaccgag 8400
8401 ttgctcttgc ccggcgtcaa cacgggataa taccgcgcca catagcagaa ctttaaaagt 8460
8461 gctcatcatt ggaaaacgtt cttcggggcg aaaactctca aggatcttac cgctgttgag 8520
8521 atccagttcg atgtaaccca ctcgtgcacc caactgatct tcagcatctt ttactttcac 8580
8581 cagcgtttct gggtgagcaa aaacaggaag gcaaaatgcc gcaaaaaagg gaataagggc 8640
8641 gacacggaaa tgttgaatac tcatactctt cctttttcaa tattattgaa gcatttatca 8700
8701 gggttattgt ctcatgagcg gatacatatt tgaatgtatt tagaaaaata aacaaatagg 8760
8761 ggttccgcgc acatttcccc gaaaagtgcc acctgacgtc taagaaacca ttattatcat 8820
8821 gacattaacc tataaaaata ggcgtatcac gaggcccttt cgtcttcaaG AATTAATTCT 8880
8881 CATGTTTGAC AGCTTATCAT CGATAAGCTG ACTCATGTTG GTATTGTGAA ATAGACGCAG 8940
8941 ATCGGGAACA CTGAAAAATA ACAGTTATTA TTCG                           8974
        |   10     |   20     |   30     |   40     |   50     |   60
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FIG.18C

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      |   10      |   20      |   30      |   40      |   50      |   60
    1 AGATCTAACA TCCAAAGACG AAAGGTTGAA TGAAACCTTT TTGCCATCCG ACATCCACAG 60
   61 GTCCATTCTC ACACATAAGT GCCAAACGCA ACAGGAGGGG ATACACTAGC AGCAGACCGT 120
  121 TGCAAACGCA GGACCTCCAC TCCTCTTCTC CTCAACACCC ACTTTTGCCA TCGAAAAACC 180
  181 AGCCCAGTTA TTGGGCTTGA TTGGAGCTCG CTCATTCCAA TTCCTTCTAT TAGGCTACTA 240
  241 ACACCATGAC TTTATTAGCC TGTCTATCCT GGCCCCCCTG GCGAGGtTCA TGTTTGTTTA 300
  301 TTTCCGAATG CAACAAGCTC CGCATTACAC CCGAACATCA CTCCAGATGA GGGCTTTCTG 360
  361 AGTGTGGGGT CAAATAGTTT CATGTTCCCc AAATGGCCCA AAACTGACAG TTTAAACGCT 420
  421 GTCTTGGAAC CTAATATGAC AAAAGCGTGA TCTCATCCAA GATGAACTAA GTTTGGTTCG 480
  481 TTGAAATGCT AACGGCCAGT TGGTCAAAAA GAAACTTCCA AAAGTCGCCA TACCGTTTGT 540
  541 CTTGTTTGGT ATTGATTGAC GAATGCTCAA AAATAATCTC ATTAATGCTT AGCGCAGTCT 600
  601 CTCTATCGCT TCTGAACCCG GTGGCACCTG TGCCGAAACG CAAATGGGGA AACaCCGCT 660
  661 TTTTGGATGA TTATGCATTG TcTCCACATT GTATGCTTCC AAGATTCTGG TGGGAATACT 720
  721 GCTGATAGCC TAACGTTCAT GATCAAAATT TAACTGTTCT AACCCCTACT TGACAGcAAT 780
  781 ATATAAACAG AAGGAAGCTG CCCTGTCTTA AACCTTTTTT TTTATCATCA TTATTAGCTT 840
  841 ACTTTCATAA TTGCGACTGG TTCCAATTGA CAAGCTTTTG ATTTTAACGA CTTTTAACGA 900
  901 CAACTTGAGA AGATCAAAAA ACAACTAATT ATTCGAAGGA TCCAAACGat gAGATTTCCT 960
  961 TCAATTTTTA CTGCAGTTTT ATTCGCAGCA TCCTCCGCAT TAGCTGCTCC AGTCAACACT 1020
 1021 ACAACAGAAG ATGAAACGGC ACAAATTCCG GCTGAAGCTG TCATCGGTTA CTCAGATTTA 1080
 1081 GAAGGGGATT TCGATGTTGC TGTTTTGCCA TTTTCCAACA GCACAAATAA CGGGTTATTG 1140
 1141 TTTATAAATA CTACTATTGC CAGCATTGCT GCTAAAGAAG AAGGGGTATC TCTCGAGAAA 1200
 1201 AGAGAGGCTG AAGCTTACGT AGAATTCgac gtcactttgt acggtactat taaggctggt 1260
 1261 gttgagactt cccgctctgt atttcaccag aacggccaag ttactgaagt tacaaccgct 1320
 1321 accggcatcg ttgatttggg ttcgaaaatc ggcttcaaag gccaagaaga cctcggtaac 1380
 1381 ggcctgaaag ccatttggca ggttgagcaa aaagcatcta tcgccggtac tgactccggt 1440
 1441 tggggcaacc gccaatcctt catcggcttg aaaggcggct tcggtaaatt gcgcgtcggt 1500
 1501 cgtttgaaca gcgtcctgaa agacaccggc gacatcaatc cttgggatag caaaagcgac 1560
 1561 tatttgggtg taaacaaaat tgccgaaccc gaggcacgcc tcatttccgt acgctacgat 1620
 1621 tctcccgaat ttgccggcct cagcggcagc gtacaatacg cgcttaacga caatgcaggc 1680
 1681 agacataaca gcgaatctta ccacgccggc ttcaactaca aaaacggtgg cttcttcgtg 1740
 1741 caatatggcg gtgcctataa aagacatcat caagtgcaag agggcttgaa tattgagaaa 1800
 1801 taccagattc accgtttggt cagcggttac gacaatgatg ccctgtacgc ttccgtagcc 1860
 1861 gtacagcaac aagacgcgaa actgactgat gcttccaatt cgcacaactc tcaaaccgaa 1920
 1921 gttgccgcta ccttggcata ccgcttcggc aacgtaacgc cccgagtttc ttacgcccac 1980
 1981 ggcttcaaag gtttggttga tgatgcagac ataggcaacg aatacgacca agtggttgtc 2040
 2041 ggtgcggaat acgacttctc caaacgcact tctgccttgg tttctgccgg ttggttgcaa 2100
 2101 gaaggcaaag gcgaaaacaa attcgtagcg actgccggcg gtgtcggtct gcgccacaaa 2160
 2161 ttctaaCCTA GGGCGGCCGC GAATTAATTC GCCTTAGACA TGACTGTTCC TCAGTTCAAG 2220
 2221 TTGGGCAcTT ACGAGAAGAC CGGTCTTGCT AGATTCTAAT CAAGAGGATG TCAGAATGCC 2280
 2281 ATTTGCCTGA GAGATGCAGG CTTCATTTTT GATACTTTTT TATTTGTAAC CTATATAGTA 2340
 2341 TAGGATTTTT TTTGTCATTT TGTTTCTTCT CGTACGAGCT TGCTCCTGAT CAGCCTATCT 2400
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FIG.19A

2401 CGCAGCTGAT GAATATCTTG TGGTAGGGGT TTGGGAAAAT CATTCGAGTT TGATGTTTTT 2460
2461 CTTGGTATTT CCCACTCCTC TTCAGAGTAC AGAAGATTAA GTGAGAAGTT CGTTTGTGCA 2520
2521 AGCTTATCGA TAAGCTTTAA TGCGGTAGTT TATCACAGTT AAATTGCTAA CGCAGTCAGG 2580
2581 CACCGTGTAT GAAATCTAAC AATGCGCTCA TCGTCATCCT CGGCACCGTC ACCCTGGATG 2640
2641 CTGTAGGCAT AGGCTTGGTT ATGCCGGTAC TGCCGGGCCT CTTGCGGGAT ATCGTCCATT 2700
2701 CCGACAGCAT CGCCAGTCAC TATGGCGTGC TGCTAGCGCT ATATGCGTTG ATGCAATTTC 2760
2761 TATGCGCACC CGTTCTCGGA GCACTGTCCG ACCGCTTTGG CCGCCGCCCA GTCCTGCTCG 2820
2821 CTTCGCTACT TGGAGCCACT ATCGACTACG CGATCATGGC GACCACACCC GTCCTGTGGA 2880
2881 TCTATCGAAT CTAAATGTAA GTTAAAATCT CTAAATAATT AAATAAGTCC CAGTTTCTCC 2940
2941 ATACGAACCT TAACAGCATT GCGGTGAGCA TCTAGACCTT CAACAGCAGC CAGATCCATC 3000
3001 ACTGCTTGGC CAATATGTTT CAGTCCCTCA GGAGTTACGT CTTGTGAAGT GATGAACTTC 3060
3061 TGGAAGGTTG CAGTGTTAAC TCCGCTGTAT TGACGGGCAT ATCCGTACGT TGGCAAAGTG 3120
3121 TGGTTGGTAC CGGAGGAGTA ATCTCCACAA CTCTCTGGAG AGTAGGCACC AACAAACACA 3180
3181 GATCCAGCGT GTTGTACTTG ATCAACATAA GAAGAAGCAT TCTCGATTTG CAGGATCAAG 3240
3241 TGTTCAGGAG CGTACTGATT GGACATTTCC AAAGCCTGCT CGTAGGTTGC AACCGATAGG 3300
3301 GTTGTAGAGT GTGCAATACA CTTGCGTACA ATTTCAACCC TTGGCAACTG CACAGCTTGG 3360
3361 TTGTGAACAG CATCTTCAAT TCTGGCAAGC TCCTTGTCTG TCATATCGAC AGCCAACAGA 3420
3421 ATCACCTGGG AATCAATACC ATGTTCAGCT TGAGACAGAA GGTCTGAGGC AACGAAATCT 3480
3481 GGATCAGCGT ATTTATCAGC AATAACTAGA ACTTCAGAAG GCCCAGCAGG CATGTCAATA 3540
3541 CTACACAGGG CTGATGTGTC ATTTTGAACC ATCATCTTGG CAGCAGTAAC GAACTGGTTT 3600
3601 CCTGGACCAA ATATTTTGTC ACACTTAGGA ACAGTTTCTG TTCCGTAAGC CATAGCAGCT 3660
3661 ACTGCCTGGG CGCCTCCTGC TAGCACGATA CACTTAGCAC CAACCTTGTG GGCAACGTAG 3720
3721 ATGACTTCTG GGGTAAGGGT ACCATCCTTC TTAGGTGGAG ATGCAAAAAC AATTTCTTTG 3780
3781 CAACCAGCAA CTTTGGCAGG AACACCCAGC ATCAGGGAAG TGGAAGGCAG AATTGCGGTT 3840

FIG.19A-1

3841 CCACCAGGAA TATAGAGGCC AACTTTCTCA ATAGGTCTTG CAAAACGAGA GCAGACTACA 3900
3901 CCAGGGCAAG TCTCAACTTG CAACGTCTCC GTTAGTTGAG CTTCATGGAA TTTCCTGACG 3960
3961 TTATCTATAG AGAGATCAAT GGCTCTCTTA ACGTTATCTG GCAATTGCAT AAGTTCCTCT 4020
4021 GGGAAAGGAG CTTCTAACAC AGGTGTCTTC AAAGCGACTC CATCAAACTT GGCAGTTAGT 4080
4081 TCTAAAAGGG CTTTGTCACC ATTTTGACGA ACATTGTCGA CAATTGGTTT GACTAATTCC 4140
4141 ATAATCTGTT CCGTTTTCTG GATAGGACGA CGAAGGGCAT CTTCAATTTC TTGTGAGGAG 4200
4201 GCCTTAGAAA CGTCAATTTT GCACAATTCA ATACGACCTT CAGAAGGGAC TTCTTTAGGT 4260
4261 TTGGATTCTT CTTTAGGTTG TTCCTTGGTG TATCCTGGCT TGGCATCTCC TTTCCTTCTA 4320
4321 GTGACCTTTA GGGACTTCAT ATCCAGGTTT CTCTCCACCT CGTCCAACGT CACACCGTAC 4380
4381 TTGGCACATC TAACTAATGC AAAATAAAAT AAGTCAGCAC ATTCCCAGGC TATATCTTCC 4440
4441 TTGGATTTAG CTTCTGCAAG TTCATCAGCT TCCTCCCTAA TTTTAGCGTT CAACAAAACT 4500
4501 TCGTCGTCAA ATAACCGTTT GGTATAAGAA CCTTCTGGAG CATTGCTCTT ACGATCCCAC 4560
4561 AAGGTGGCTT CCATGGCTCT AAGACCCTTT GATTGGCCAA AACAGGAAGT GCGTTCCAAG 4620
4621 TGACAGAAAC CAACACCTGT TTGTTCAACC ACAAATTTCA AGCAGTCTCC ATCACAATCC 4680
4681 AATTCGATAC CCAGCAACTT TTGAGTTGCT CCAGATGTAG CACCTTTATA CCACAAACCG 4740
4741 TGACGACGAG ATTGGTAGAC TCCAGTTTGT GTCCTTATAG CCTCCGGAAT AGACTTTTTG 4800
4801 GACGAGTACA CCAGGCCCAA CGAGTAATTA GAAGAGTCAG CCACCAAAGT AGTGAATAGA 4860
4861 CCATCGGGGC GGTCAGTAGT CAAAGACGCC AACAAAATTT CACTGACAGG GAACTTTTTG 4920
4921 ACATCTTCAG AAAGTTCGTA TTCAGTAGTC AATTGCCGAG CATCAATAAT GGGGATTATA 4980
4981 CCAGAAGCAA CAGTGGAAGT CACATCTACC AACTTTGCGG TCTCAGAAAA AGCATAAACA 5040
5041 GTTCTACTAC CGCCATTAGT GAAACTTTTC AAATCGCCCA GTGGAGAAGA AAAAGGCACA 5100
5101 GCGATACTAG CATTAGCGGG CAAGGATGCA ACTTTATCAA CCAGGGTCCT ATAGATAACC 5160
5161 CTAGCGCCTG GGATCATCCT TTGGACAACT CTTTCTGCCA AATCTAGGTC CAAAATCACT 5220
5221 TCATTGATAC CATTATTGTA CAACTTGAGC AAGTTGTCGA TCAGCTCCTC AAATTGGTCC 5280
5281 TCTGTAACGG ATGACTCAAC TTGCACATTA ACTTGAAGCT CAGTCGATTG AGTGAACTTG 5340
5341 ATCAGGTTGT GCAGCTGGTC AGCAGCATAG GGAAACACGG CTTTTCCTAC CAAACTCAAG 5400
5401 GAATTATCAA ACTCTGCAAC ACTTGCGTAT GCAGGTAGCA AGGGAAATGT CATACTTGAA 5460
5461 GTCGGACAGT GAGTGTAGTC TTGAGAAATT CTGAAGCCGT ATTTTTATTA TCAGTGAGTC 5520
5521 AGTCATCAGG AGATCCTCTA CGCCGGACGC ATCGTGGCCG ACCTGCAGGg gggggggggG 5580
5581 CGCTGAGGTC TGCCTCGTGA AGAAGGTGTT GCTGACTCAT ACCAGGCCTG AATCGCCCCA 5640
5641 TCATCCAGCC AGAAAGTGAG GGAGCCACGG TTGATGAGAG CTTTGTTGTA GGTGGACCAG 5700
5701 TTGGTGATTT TGAACTTTTG CTTTGCCACG GAACGGTCTG CGTTGTCGGG AAGATGCGTG 5760
5761 ATCTGATCCT TCAACTCAGC AAAAGTTCGA TTTATTCAAC AAAGCCGCCG TCCCGTCAAG 5820
5821 TCAGCGTAAT GCTCTGCCAG TGTTACAACC AATTAACCAA TTCTGATTAG AAAAACTCAT 5880
5881 CGAGCATCAA ATGAAACTGC AATTTATTCA TATCAGGATT ATCAATACCA TATTTTTGAA 5940
5941 AAAGCCGTTT CTGTAATGAA GGAGAAAACT CACCGAGGCA GTTCCATAGG ATGGCAAGAT 6000
6001 CCTGGTATCG GTCTGCGATT CCGACTCGTC CAACATCAAT ACAACCTATT AATTTCCCCT 6060
6061 CGTCAAAAAT AAGGTTATCA AGTGAGAAAT CACCATGAGT GACGACTGAA TCCGGTGAGA 6120
6121 ATGGCAAAAG CTTATGCATT TCTTTCCAGA CTTGTTCAAC AGGCCAGCCA TTACGCTCGT 6180
6181 CATCAAAATC ACTCGCATCA ACCAAACCGT TATTCATTCG TGATTGCGCC TGAGCGAGAC 6240
6241 GAAATACGCG ATCGCTGTTA AAAGGACAAT TACAAACAGG AATCGAATGC AACCGGCGCA 6300
6301 GGAACACTGC CAGCGCATCA ACAATATTTT CACCTGAATC AGGATATTCT TCTAATACCT 6360
6361 GGAATGCTGT TTTCCCGGGG ATCGCAGTGG TGAGTAACCA TGCATCATCA GGAGTACGGA 6420
6421 TAAAATGCTT GATGGTCGGA AGAGGCATAA ATTCCGTCAG CCAGTTTAGT CTGACCATCT 6480
6481 CATCTGTAAC ATCATTGGCA ACGCTACCTT TGCCATGTTT CAGAAACAAC TCTGGCGCAT 6540

6541 CGGGCTTCCC ATACAATCGA TAGATTGTCG CACCTGATTG CCCGACATTA TCGCGAGCCC 6600
6601 ATTTATACCC ATATAAATCA GCATCCATGT TGGAATTTAA TCGCGGCCTC GAGCAAGACG 6660
6661 TTTCCCGTTG AATATGGCTC ATAACACCCC TTGTATTACT GTTTATGTAA GCAGACAGTT 6720
6721 TTATTGTTCA TGATGATATA TTTTTATCTT GTGCAATGTA ACATCAGAGA TTTTGAGACA 6780
6781 CAACGTGGCT TTCCCCCCCC CCCCTGCAGG TCGGCATCAC CGGCGCCACA GGTGCGGTTG 6840
6841 CTGGCGCCTA TATCGCCGAC ATCACCGATG GGGAAGATCG GGCTCGCCAC TTCGGGCTCA 6900
6901 TGAGCGCTTG TTTCGGCGTG GGTATGGTGG CAGGCCCCGT GGCCGGGGGA CTGTTGGGCG 6960
6961 CCATCTCCTT GCATGCACCA TTCCTTGCGG CGGCGGTGCT CAACGGCCTC AACCTACTAC 7020
7021 TGGGCTGCTT CCTAATGCAG GAGTCGCATA AGGGAGAGCG TCGAGTATCT ATGATTGGAA 7080
7081 GTATGGGAAT GGTGATACCC GCATTCTTCA GTGTCTTGAG GTCTCCTATC AGATTATGCC 7140
7141 CAACTAAAGC AACCGGAGGA GGAGATTTCA TGGTAAATTT CTCTGACTTT TGGTCATCAG 7200
7201 TAGACTCGAA CTGTGAGACT ATCTCGGTTA TGACAGCAGA AATGTCCTTC TTGGAGACAG 7260
7261 TAAATGAAGT CCCACCAATA AAGAAATCCT TGTTATCAGG AACAAACTTC TTGTTTCGAA 7320
7321 CTTTTTCGGT GCCTTGAACT ATAAAATGTA GAGTGGATAT GTCGGGTAGG AATGGAGCGG 7380
7381 GCAAATGCTT ACCTTCTGGA CCTTCAAGAG GTATGTAGGG TTTGTAGATA CTGATGCCAA 7440
7441 CTTCAGTGAC AACGTTGCTA TTTCGTTCAA ACCATTCCGA ATCCAGAGAA ATCAAAGTTG 7500
7501 TTTGTCTACT ATTGATCCAA GCCAGTGCGG TCTTGAAACT GACAATAGTG TGCTCGTGTT 7560
7561 TTGAGGTCAT CTTTGTATGA ATAAATCTAG TCTTTGATCT AAATAATCTT GACGAGCCAA 7620
7621 GGCGATAAAT ACCCAAATCT AAAACTCTTT TAAAACGTTA AAAGGACAAG TATGTCTGCC 7680
7681 TGTATTAAAC CCCAAATCAG CTCGTAGTCT GATCCTCATC AACTTGAGGG GCACTATCTT 7740
7741 GTTTTAGAGA AATTTGCGGA GATGCGATAT CGAGAAAAAG GTACGCTGAT TTTAAACGTG 7800
7801 AAATTTATCT CAAGATCTCT GCCTCGCGCG TTTCGGTGAT GACGGTGAAA ACCTCTGACA 7860
7861 CATGCAGCTC CCGGAGACGG TCACAGCTTG TCTGTAAGCG GATGCCGGGA GCAGACAAGC 7920
7921 CCGTCAGGGC GCGTCAGCGG GTGTTGGCGG GTGTCGGGGC GCAGCCATGA CCCAGTCACG 7980
7981 TAGCGATAGC GGAGTGTATA CTGGCTTAAC TATGCGGCAT CAGAGCAGAT TGTACTGAGA 8040
8041 GTGCACCATA TGCGGTGTGA AATACCGCAC AGATGCGTAA GGAGAAAATA CCGCATCAGG 8100
8101 CGCTCTTCCG CTTCCTCGCT CACTGACTCG CTGCGCTCGG TCGTTCGGCT GCGGCGAGCG 8160

FIG.19B-1

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8161 GTATCAGCTC ACTCAAAGGC GGTAATACGG TTATCCACAG AATCAGGGGA TAACGCAGGA 8220
8221 AAGAACATGT GAGCAAAAGG CCAGCAAAAG GCCAGGAACC GTAAAAAGGC CGCGTTGCTG 8280
8281 GCGTTTTTCC ATAGGCTCCG CCCCCCTGAC GAGCATCACA AAAATCGACG CTCAAGTCAG 8340
8341 AGGTGGCGAA ACCCGACAGG ACTATAAAGA TACCAGGCGT TTCCCCCTGG AAGCTCCCTC 8400
8401 GTGCGCTCTC CTGTTCCGAC CCTGCCGCTT ACCGGATACC TGTCCGCCTT TCTCCCTTCG 8460
8461 GGAAGCGTGG CGCTTTCTCA ATGCTCACGC TGTAGGTATC TCAGTTCGGT GTAGGTCGTT 8520
8521 CGCTCCAAGC TGGGCTGTGT GCACGAACCC CCCGTTCAGC CCGACCGCTG CGCCTTATCC 8580
8581 GGTAACTATC GTCTTGAGTC CAACCCGGTA AGACACGACT TATCGCCACT GGCAGCAGCC 8640
8641 ACTGGTAACA GGATTAGCAG AGCGAGGTAT GTAGGCGGTG CTACAGAGTT CTTGAAGTGG 8700
8701 TGGCCTAACT ACGGCTACAC TAGAAGGACA GTATTTGGTA TCTGCGCTCT GCTGAAGCCA 8760
8761 GTTACCTTCG GAAAAAGAGT TGGTAGCTCT TGATCCGGCA AACAAACCAC CGCTGGTAGC 8820
8821 GGTGGTTTTT TTGTTTGCAA GCAGCAGATT ACGCGCAGAA AAAAAGGATC TCAAGAAGAT 8880
8881 CCTTTGATCT TTTCTACGGG GTCTGACGCT CAGTGGAACG AAAACTCACG TTAAGGGATT 8940
8941 TTGGTCATGA GATTATCAAA AAGGATCTTC ACCTAGATCC TTTTAAATTA AAAATGAAGT 9000
9001 TTTAAATCAA TCTAAAGTAT ATATGAGTAA ACTTGGTCTG ACAGTTACCA ATGCTTAATC 9060
9061 AGTGAGGCAC CTATCTCAGC GATCTGTCTA TTTCGTTCAT CCATAGTTGC CTGACTCCCC 9120
9121 GTCGTGTAGA TAACTACGAT ACGGGAGGGC TTACCATCTG GCCCCAGTGC TGCAATGATA 9180
9181 CCGCGAGACC CACGCTCACC GGCTCCAGAT TTATCAGCAA TAAACCAGCC AGCCGGAAGG 9240
9241 GCCGAGCGCA GAAGTGGTCC TGCAACTTTA TCCGCCTCCA TCCAGTCTAT TAATTGTTGC 9300
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9361 GCAGGCATCG TGGTGTCACG CTCGTCGTTT GGTATGGCTT CATTCAGCTC CGGTTCCCAA 9420
9421 CGATCAAGGC GAGTTACATG ATCCCCCATG TTGTGCAAAA AAGCGGTTAG CTCCTTCGGT 9480
9481 CCTCCGATCG TTGTCAGAAG TAAGTTGGCC GCAGTGTTAT CACTCATGGT TATGGCAGCA 9540
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9601 TCAACCAAGT CATTCTGAGA ATAGTGTATG CGGCGACCGA GTTGCTCTTG CCCGGCGTCA 9660
9661 ACACGGGATA ATACCGCGCC ACATAGCAGA ACTTTAAAAG TGCTCATCAT TGGAAAACGT 9720
9721 TCTTCGGGGC GAAAACTCTC AAGGATCTTA CCGCTGTTGA GATCCAGTTC GATGTAACCC 9780
9781 ACTCGTGCAC CCAACTGATC TTCAGCATCT TTTACTTTCA CCAGCGTTTC TGGGTGAGCA 9840
9841 AAAACAGGAA GGCAAAATGC CGCAAAAAAG GGAATAAGGG CGACACGGAA ATGTTGAATA 9900
9901 CTCATACTCT TCCTTTTTCA ATATTATTGA AGCATTTATC AGGGTTATTG TCTCATGAGC 9960
9961 GGATACATAT TTGAATGTAT TTAGAAAAAT AAACAAATAG GGGTTCCGCG CACATTTCCC 10020
10021 CGAAAAGTGC CACCTGACGT CTAAGAAACC ATTATTATCA TGACATTAAC CTATAAAAAT 10080
10081 AGGCGTATCA CGAGGCCCTT TCGTCTTCAA GAATTAATTC TCATGTTTGA CAGCTTATCA 10140
10141 TCGATAAGCT GACTCATGTT GGTATTGTGA AATAGACGCA GATCGGGAAC ACTGAAAAAT 10200
10201 AACAGTTATT ATTCG                                                  10215
          |  10      |  20      |  30      |  40      |  50      |  60
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FIG.19C

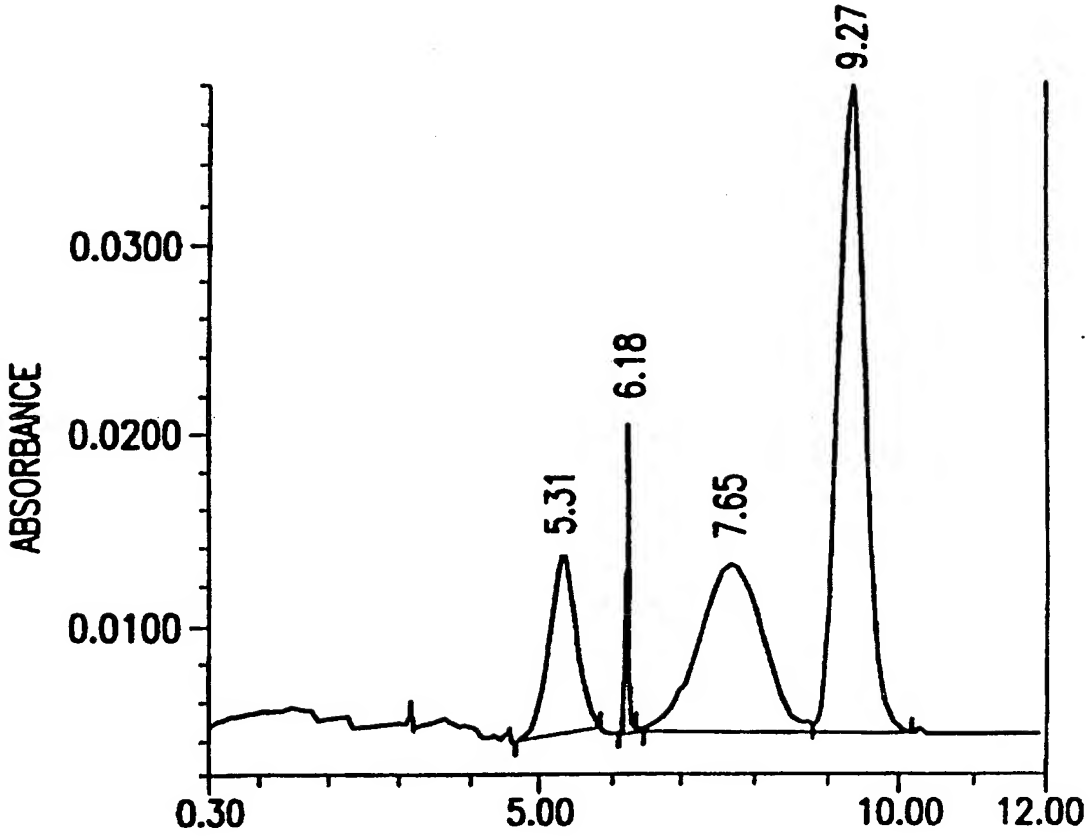

FIG.20

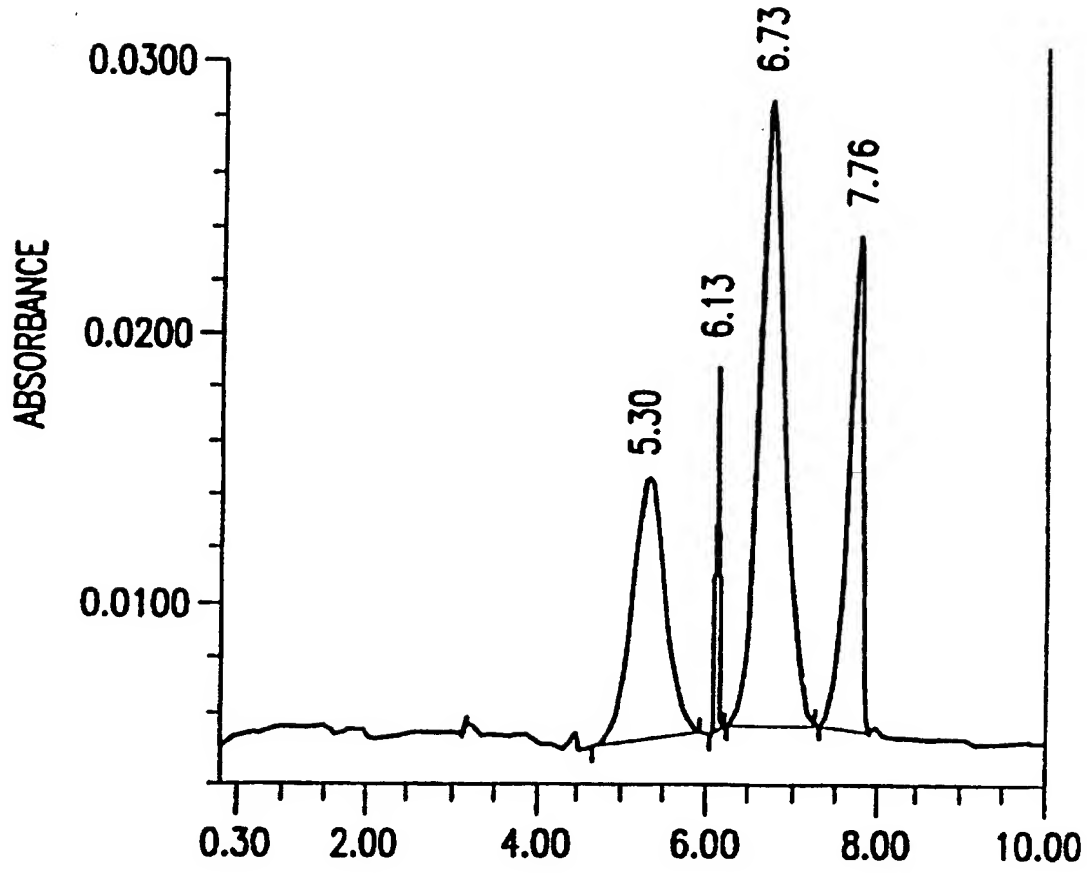

FIG.21

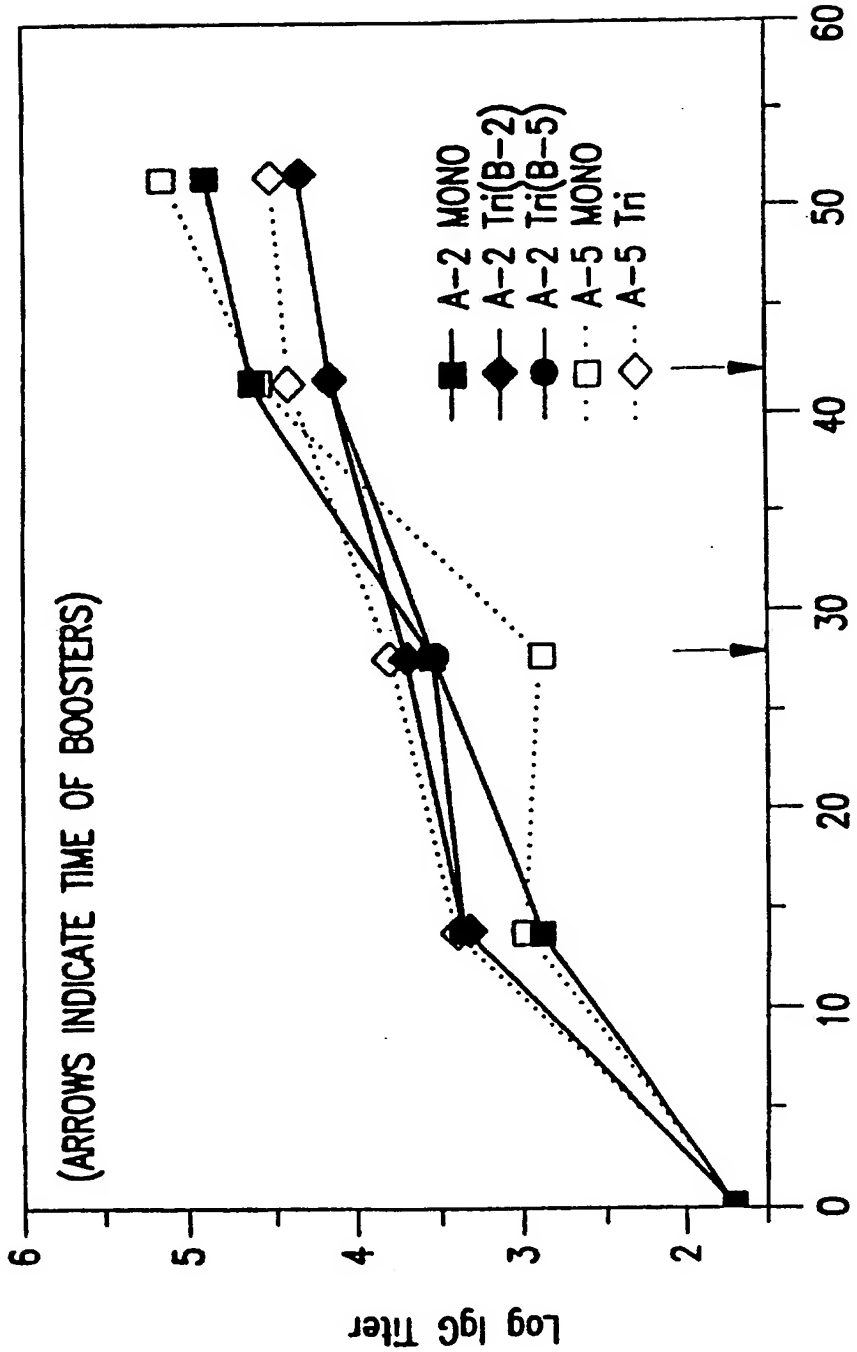

FIG.22

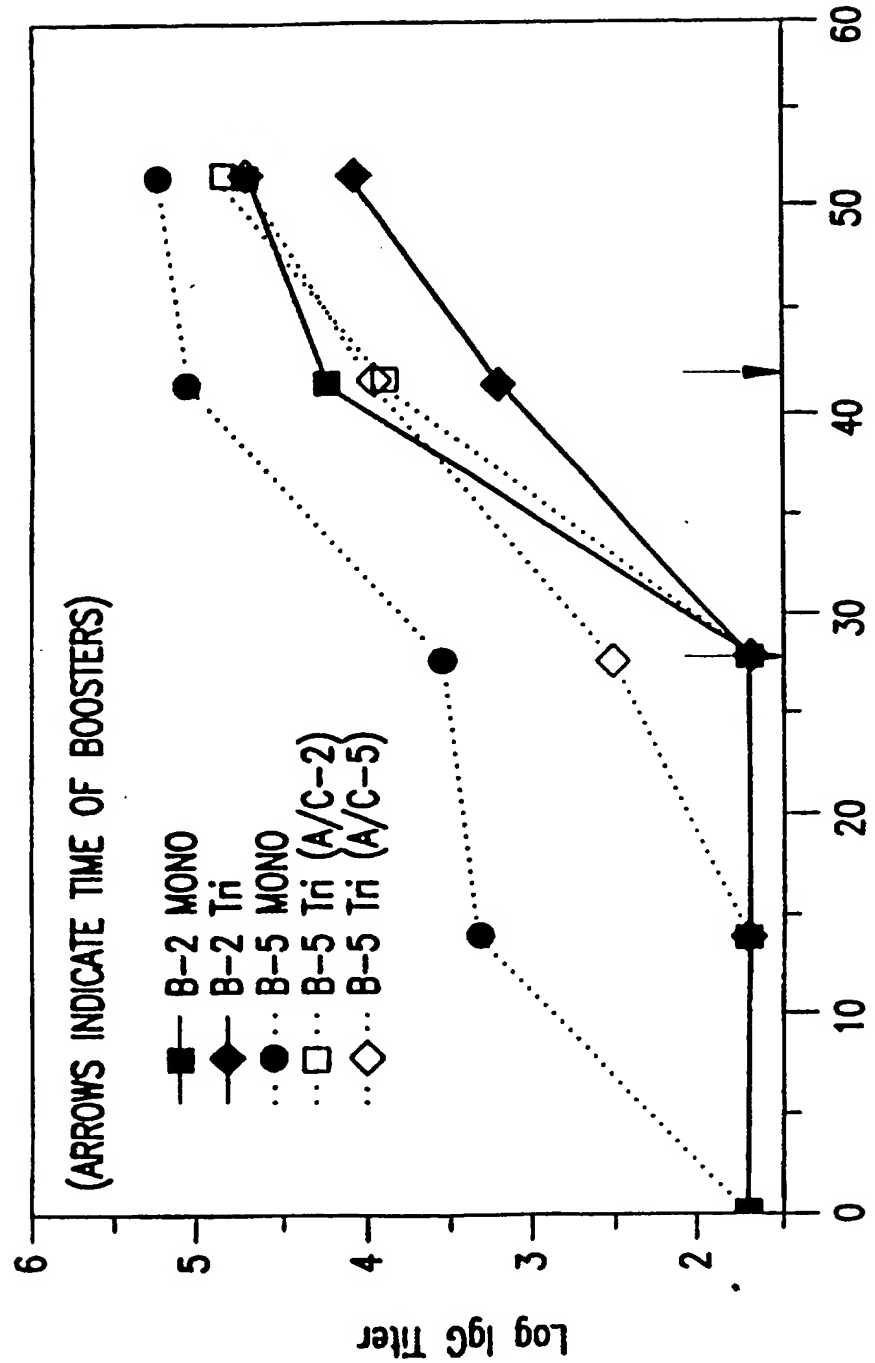

FIG.23

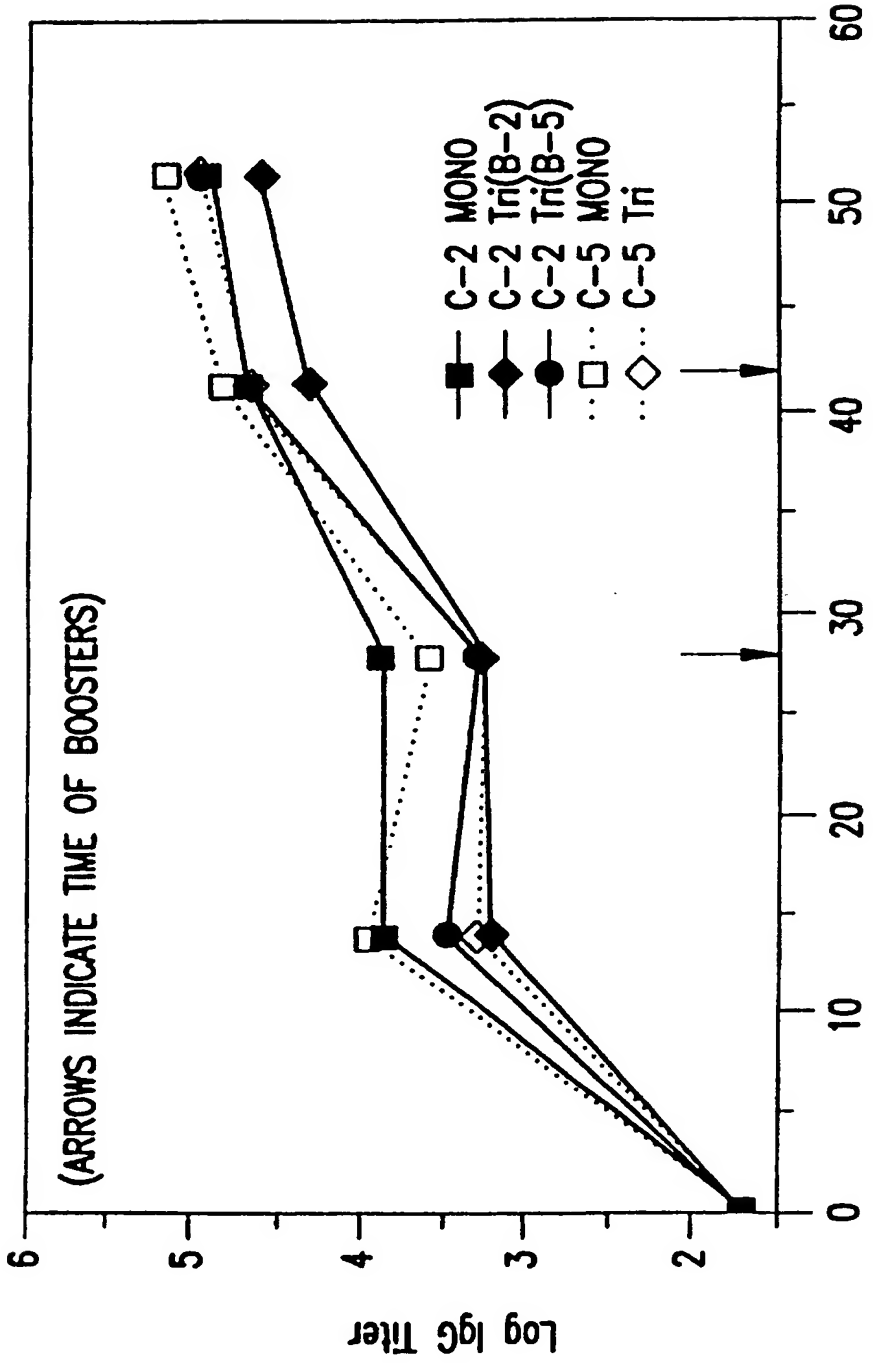

FIG.24

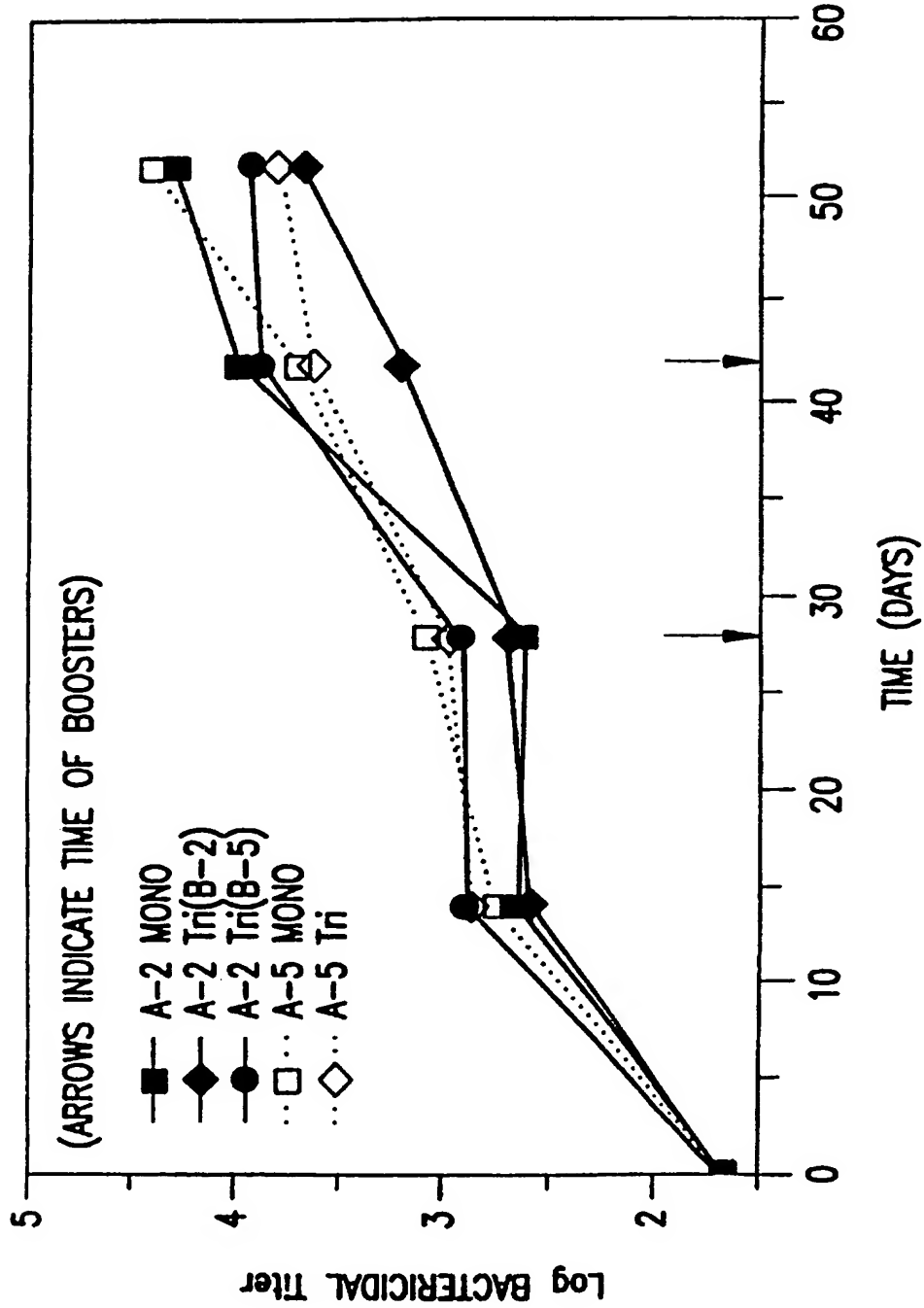

FIG.25

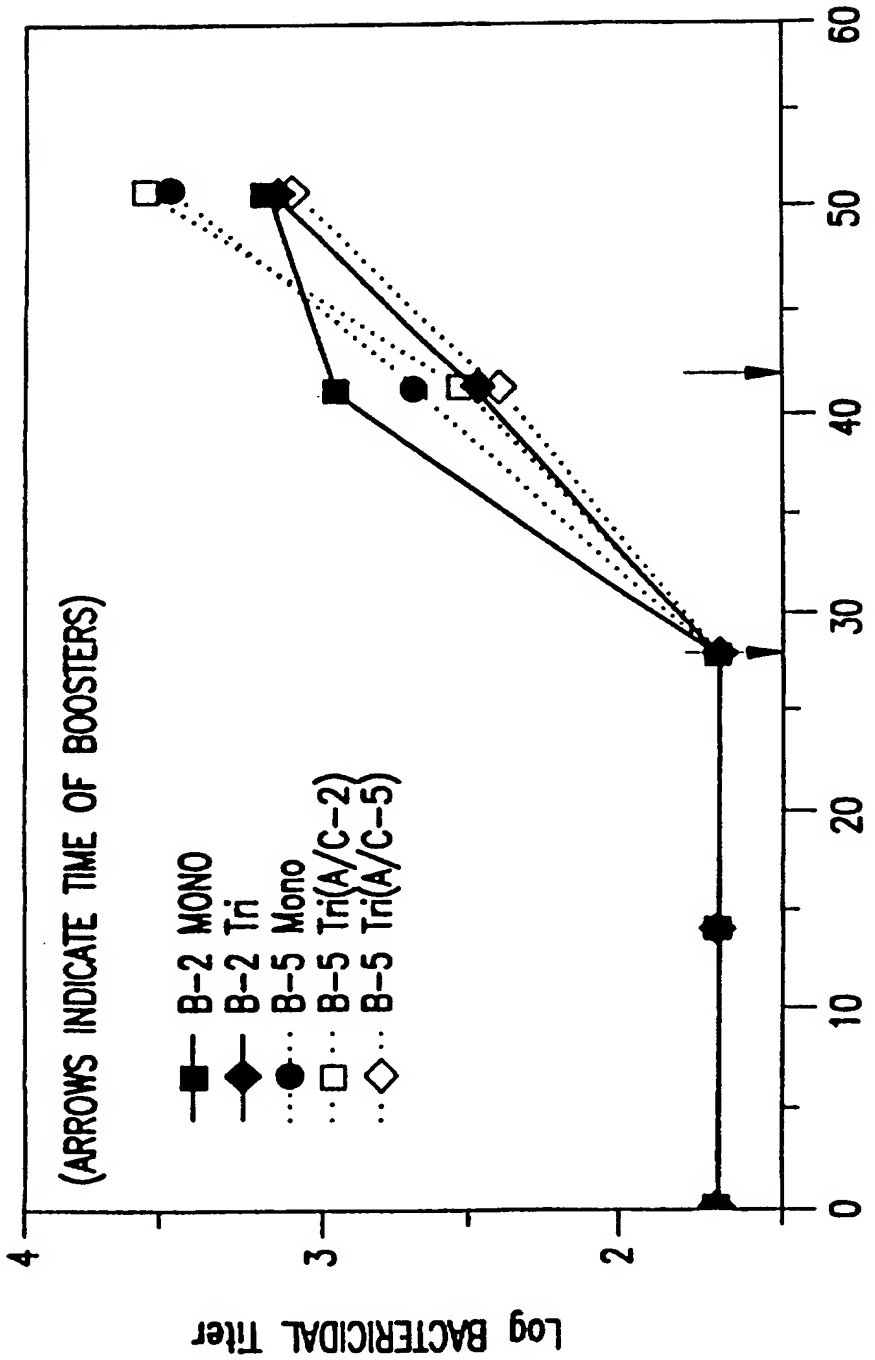

FIG.26

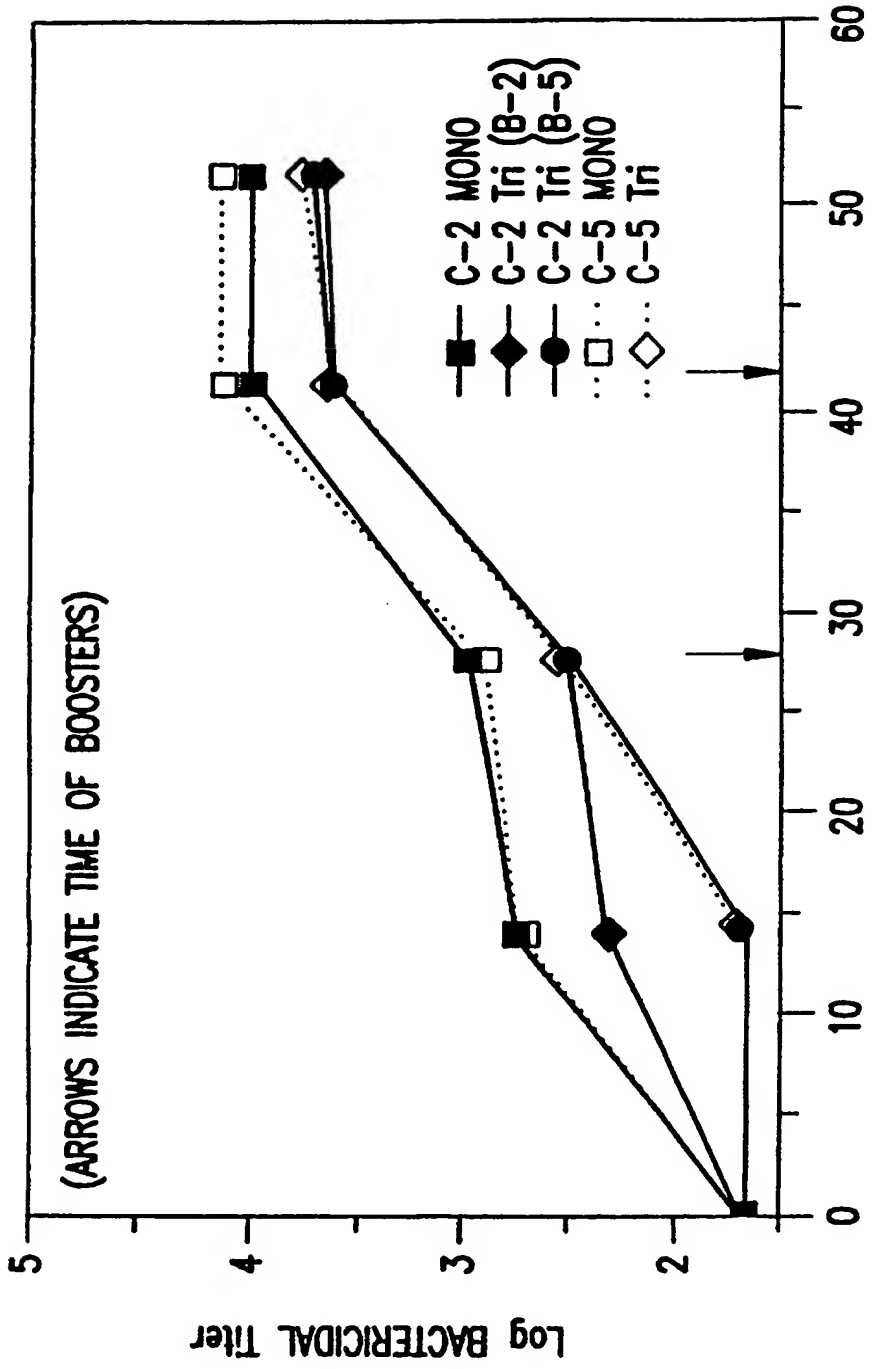

FIG.27

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/01687

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/69.1, 69.7, 69.8, 69.9, 255.1, 320.1; 530/412, 416, 417; 536/23.7; 424/185.1, 192.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, CA, EMBASE, WPIDS
terms: meningococcal, porin, expression, group A, B, and C, pastoris, wobble

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 4,356,170 A (JENNINGS et al.) 26 October 1982 (26/10/82), see entire document.	23, 24, 26, 28-32 --- 25, 27, 33
Y	WO 95/03413 A1 (THE ROCKEFELLER UNIVERSITY) 02 February 1995 (02.02.95), see entire document.	1-33
Y	US 5,268,273 A (BUCKHOLZ) 07 December 1993 (07/12/93), see entire document.	1-22

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 08 MAY 1997	Date of mailing of the international search report 11 JUL 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer I W for MARK NAVARRO Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/01687

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BLACHLY-DYSON et al. Cloning and Functional Expression in Yeast of Two Human Isoforms of the Outer Mitochondrial Membrane Channel, the Voltage-dependent Anion Channel. Journal of Biological Chemistry. 25 January 1993 Vol. 268, No. 3, pages 1835-1841.	1-22
Y	CHOI et al. Study of Putative Glycosylation Sites in Bovine β-Casein Introduced by PCR-Based Site-Directed Mutagenesis. J. Agric. Food Chem.. January 1996, Vol. 44, No. 1, pages 358-364.	1-22
Y	BENNETZEN et al. Codon Selection in Yeast. Journal of Biological Chemistry. 25 March 1982, Vol. 257, No. 6, pages 3026-3031.	1-22
Y	MITRA. YEAST tRNA (ANTICODON CUU) TRANSLATES AAA CODON. FEBS Letters. July 1978, Volumn 91, Number 1, pages 78-80, see entire document.	1-22
Y	HALSTENSEN et al. Human Opsonins to Meningococci After Vaccination. Infection and Immunity. December 1984, Vol. 46, No. 3, pages 673-676, see entire document.	23-33
Y	WO 92/04915 A1 (NORTH AMERICAN VACCINE, INC.) 02 April 1992 (02.04.92), see entire document.	23-33
Y	JENNINGS et al. Induction of Meningococcal Group B Polysaccharide-Specific IgG Antibodies In Mice By Using An N-Propionylated B Polysaccharide-Tetanus Conjugate Vaccine. Journal of Immunology. 01 September 1986, Vol. 137, No. 5, pages 1708-1713, see entire document.	25, 27

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/01687

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C12P 21/04, 21/06; C12N 15/00, 1/14; A23J 1/00; C07K 1/00; C07H 21/04; A61K 39/00, 39/385

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/69.1, 69.7, 69.8, 69.9, 255.1, 320.1; 530/412, 416, 417; 536/23.7; 424/185.1, 192.1